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Measurement of Trypsin Activity Using a Radioactive Substrate.* (26298)

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Recently the authors reported their use of RIHSA (radioactive iodinated human serum albumin—Abbott Laboratories) as a substrate for measuring pepsin activity(1). Because of its simplicity and reliability for assaying the activity of pepsin, its value for measuring trypsin activity was also tested. The procedure was similar to that reported earlier for pepsin, except for necessary changes in pH for optimum activity of trypsin, in enzyme concentration and in method of precipitation after incubation of enzyme and substrate.

This is a report of various factors affecting trypsin activity as measured by the RIHSA, Anson(2) and Kunitz(3) methods. Spectrophotometric measurements were also made of the supernatant fluid derived from analyses with hemoglobin as substrate. This latter technic which combines features from both the Anson and Kunitz methods is referred to in the text as the "spectrophotometric" method.

Materials and methods. Lyophilized crystalline trypsin—Armour Laboratories (Tryptar®) contained 2640 units of trypsin activity per milligram. The enzyme was dissolved in one of various aqueous solutions used in these studies. Control solutions for which the solvent was deionized water or McIlvaine's Buffer, were always included.

Egg albumin (Mallinckrodt Laboratories) dissolved in distilled water at a concentration of 4 mg/ml served as the stock substrate for the RIHSA studies. Immediately before its use, this stock solution was diluted 1:1 with 0.2 M Sorenson's phosphate buffer and one microcurie of RIHSA was added for each 10 ml of solution. This mixture was then passed through an anion exchange resin column to remove radioactive iodine not bonded to albumin(4). Stable albumin was added to the substrate to promote precipitation of the undigested protein at termination of the

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TABLE I. Trypsin Stability as a Function of Solvent pH.

pH	Trypsin activity* (with stand. errors)	pH	Trypsin activity* (with stand. errors)
2.5	80 ± 5.5	5.5	79 ± 6.2
3.0	86 3.1	6.0	63 6.3
3.5	94 4.7	6.5	46 3.2
4.0	100 5.8	7.0	37 1.6
4.5	100 5.7	7.5	34 1.6
5.0	90 6.4	8.0	34 1.6

* Arbitrary units.

Time between solvation and assay of trypsin—3 hr.

Incubation pH—7.5.

incubation period. Hemoglobin and casein substrates were prepared in accordance with published methods(2,3).

A pre-determined amount of one of these substrates was added to each tube containing trypsin after which all tubes were incubated in a water bath at 37°C for one hour. After incubation instantaneous inactivation of the enzyme and precipitation of both enzyme and substrate were achieved by addition of acid. Five % tricholoracetic acid was used as specified in the Anson and Kunitz methods. For the RIHSA method, 5% sulfosalicylic acid was found to be more effective in promoting precipitation than was 5% trichloroacetic acid or other concentrations of sulfosalicylic acid.

After centrifugation, aliquots were taken for the various analyses. For the RIHSA method the radioactive iodine freed from the digested albumin molecules remained in the supernatant fluid. The gamma radiation emitted by the iodine in an aliquot of the supernatant was assayed in a well scintillation counter as a measure of enzyme activity. Analyses of the supernatant from the hemoglobin and casein substrates were made according to the methods outlined by Anson and Kunitz.

Results. Preliminary investigations using RIHSA method. Trypsin stability as a function of solvent pH was investigated by dissolving trypsin in an aqueous solution containing varying amounts of 0.005 N citric acid and 0.005 N sodium hydroxide to give a pH range from 2.5 to 8.0. These solutions were permitted to stand for 3 hours prior to incubation. In this study it was necessary to increase the final concentration of the substrate buffer to 0.2 M to maintain the incubation pH at 7.5 ± 0.2. Results are shown in Table I.

A range of pH from 5.0 to 8.5 during incubation was used to study trypsin activity as a function of its incubation pH. This was accomplished by using varying concentrations of the components of 0.2 M phosphate buffer in making up the substrate. Results indicate that the pH for optimum tryptic activity is between pH 6.5 and 7.0 (Table II).

Trypsin activity as measured with RIHSA, hemoglobin or casein substrates. The activity of trypsin *vs.* its concentration was studied by incubating samples of trypsin varying in concentration from 50 to 8000 trypsin units per ml. A comparison of the results from the RIHSA, Anson and Kunitz methods is plotted in Fig. 1.

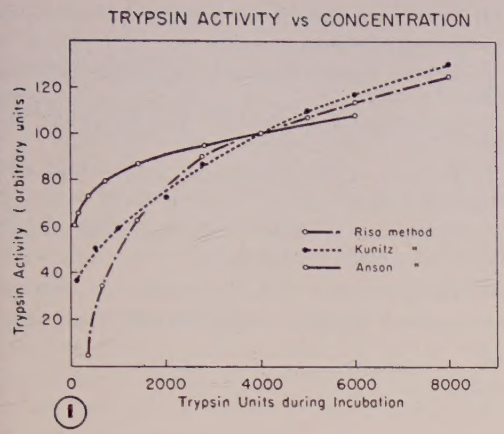
Results using the RIHSA, Anson, Kunitz and spectrophotometric methods for measuring tryptic activity at various incubation times are shown in Fig. 2. Trypsin concentration was maintained at 5280 T.U./ml for the RIHSA method and at 129 T.U./ml for the other methods.

Discussion. Our studies indicate that trypsin is most stable in solution at pH 4.5 which is near the pH for optimum stability of pepsin(1). The decrease in trypsin stability as the solvent pH approaches that for optimum activity of this enzyme is thought to be attributable to autolysis. The decrease in stability using solvents with pH values below 4.5 is possibly due to the effect of the citric acid used to control the pH. This pH for optimum trypsin stability is higher than the value of 2.0 reported by Kunitz and Northrop(5) and differs considerably from the

TABLE II. Trypsin Activity when Incubated with Substrate at Various pH Values.

pH	Trypsin activity* (with stand. errors)	pH	Trypsin activity* (with stand. errors)
5.0	50 ± 5.2	7.0	100 ± 2.7
5.5	67 3.8	7.5	94 4.3
6.0	86 2.8	8.0	80 4.2
6.5	100 1.9	8.5	61 5.6

* Arbitrary units.



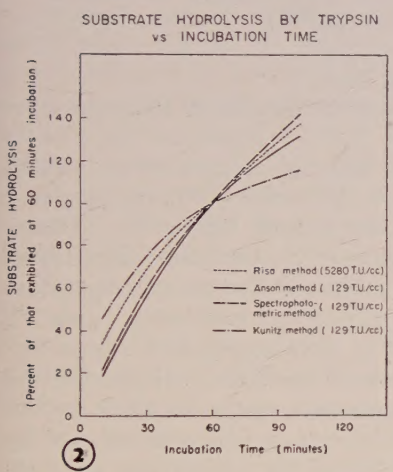


FIG. 1. Trypsin activity as a function of its concentration as measured by three methods.

FIG. 2. Substrate hydrolysis as a function of incubation time as measured by four methods.

work of Crewther(6) who found peaks of trypsin stability at pH 2.9, 6.6 and 10.7. It is possible that differences in substrates used for assay of trypsin activity may account for the greater part of these apparent discrepancies. To facilitate an evaluation of relative merits of these various methods, we chose to standardize the incubation pH at 7.6 for all studies in which these comparisons were being made.

In both the Anson and Kunitz methods the pH of the substrate used for assay of trypsin activity is maintained at 7.6.

Using 5% casein as the substrate Northrop and Kunitz(7) found a flat broad curve of optimum activity extending from pH 7.5 to 9.5. Bergmann and associates(8) show a sharp domed curve with a peak at pH 7.75 employing benzoyl-l-arginineamide as a substrate. These values are both above the optimum incubation pH as determined by the RIHSA method (pH 6.5-7.0).

Fig. 1 shows that for low concentration of trypsin, the RIHSA method is not as sensitive as either the Anson or Kunitz methods. Of these methods, that of Anson appears to be the most useful for measuring very small amounts of trypsin. With concentration of trypsin above about 2000 T.U./ml small differences in enzymatic activity are most readily detectable by the Kunitz or RIHSA methods.

As the concentration of trypsin was increased above about 2000 T.U./ml the results obtained by the Kunitz and RIHSA methods show good agreement. However, because of its simplicity, the RIHSA method is likely to be preferred for measuring the activity of relatively large concentrations of trypsin. These results of trypsin activity *vs.* its concentration agree favorably with those reported by Anson(2) and Kunitz(3).

There is a gradual decrease in trypsin activity with increasing incubation time for all the methods used. This is evidenced by the decreasing slope of the hydrolysis curve (Fig. 2). This is most apparent for long incubation times using Kunitz' method in which concentration of the substrate is likely the actual limiting factor for the reaction. Good agreement is found for all methods when incubation time is restricted to one hour or less.

Summary. Comparison has been made of measurements of trypsin activity by a substrate containing RIHSA (radioactive iodinated human serum albumin—Abbott Laboratories) and the commonly used methods of Anson and Kunitz. In this method, trypsin was incubated with an albumin substrate containing RIHSA, followed by precipitation of the undigested substrate with sulfosalicylic acid and measurement of radioactive digestion products in the supernatant fluid. Trypsin was found to be most stable in solution at a pH near 4.5 using the RIHSA method. Its activity was maximal at pH 6.5-7.0. The RIHSA method did not provide as sensitive

a test for low concentrations of trypsin as did the methods of Anson and Kunitz. However, because of its simplicity for assay of tryptic activity, the RIHSA method is considered to be of definite value when enzyme concentrations to be assayed are in excess of 2000 T.U. per ml.

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Endotoxin Shock and the Coagulation Mechanism: Modification of Shock with Epsilon-Aminocaproic Acid. (26299)

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Injection of a lethal dose of endotoxin into the dog is followed by a characteristic pattern of hemodynamic changes(1,2). Within a minute there is a marked decline in systemic blood pressure and rise in portal vein pressure. Fifteen to 30 minutes later the blood pressure tends to approach pre-injection level, then a gradual decline occurs, accompanied by oliguria or anuria, acidosis, and hemoconcentration. Death takes place within 24 hours.

We have reviewed elsewhere our investigations on the mechanism of vascular alterations induced by endotoxin in the dog(2,3,4). Briefly, the initial vasoconstriction caused by endotoxin is probably due to histamine or a histamine-like substance, release of which is mediated through a component or components of blood. Contraction of an isolated canine vein occurred when the vessel was exposed to a bath containing endotoxin and fresh whole blood. Enzymatic participation in the reaction was suggested by the latent period that occurred before contraction took place; and by an activating serum factor that was dialyzable and heat-labile. Involvement of proteolytic activity was considered, possibly associated with the coagulation mechanism. Such a concept is suggested by other investigations. Unger and Mist(5) postulated that specific antigen-antibody reactions, peptones or bacterial polysaccharides caused liberation of histamine through increased proteolytic activity of the serum. Others have concluded(6,7) that the Shwartzman reaction in rabbits is a result of coagulation alterations caused by endotoxin. Deutsch and Elsner(8) have stated that administration of endotoxin results in activation of the plasminogen-plasmin system in which an initial state of hypercoagulability is followed by accelerated fibrinolysis. Gans and Krivit(9, 10) reporting on some significant comparative studies on endotoxin in rabbits and dogs as to effects on the coagulation mechanism, concluded that endotoxin did not activate the plasminogen-plasmin system in rabbits. Therefore, in this species endotoxin causes extensive thrombosis, which is a feature of the Shwartzman reaction. Endotoxin does not produce the Shwartzman reaction in the dog because plasminogen is activated and thrombolytic activity is prominent. Pertinent to the present study they found that endotoxin in the dog caused a marked increase in activity of plasminogen-activator, and a decline in plasminogen concentration.

Epsilon-aminocaproic acid (hereafter referred to as EACA) is a potent inhibitor of plasminogen activation(11). Sherry and his associates(12,13) have studied EACA intensively, both *in vitro* and in human subjects,

and have concluded that although the action of EACA on the coagulation mechanism is complex, the most striking effect is the inhibition of plasminogen activation.

If endotoxin shock in the dog is associated with activation of plasminogen, and the increase in proteolytic activity results in liberation of histamine, inhibition of plasminogen activation with EACA should lead to a reduction in severity of the vascular response to endotoxin. The present experiments demonstrate that pre-treatment of adult dogs with EACA results in survival of the majority of the animals injected with a lethal dose of endotoxin.*

Materials and methods. Endotoxin. A standardized amount of endotoxin, prepared from a strain of *Escherichia coli* according to methods described elsewhere(14), was given intravenously to dogs in a LD_{100} dose of 0.55 mg/kg. Animals expired within 24 hours from peripheral vascular collapse.

EACA. A sterile solution was slowly infused intravenously as a 1% solution in 200 ml of 5% dextrose and water.†

Animals. Adult mongrel male dogs were used in all of the experiments and housed in individual cages. Only water was permitted on day of experiments. Following each experiment surviving dogs were returned to the cages and offered food and water. "Survivals" included those animals that resumed their usual physical activities outside of the cages after recovery from the effects of endotoxin.

Experimental procedure. The dogs were anesthetized with 30 mg/kg body weight of sodium pentobarbital, and sufficient barbiturate was given subsequently to maintain a constant level of anesthesia.

Systemic arterial pressure was monitored continuously by means of a polyethylene catheter placed in the femoral artery, which in turn was connected to a Statham strain gauge, and tracings were made on a Sanborn Twin Viso recorder. The bladder was catheterized, and urine quantitated hourly. The pH of the femoral arterial blood was measured hourly with a Beckman pH meter, and hourly hematocrit determinations were also made.

Exp. 1. Twelve dogs were anesthetized and control observations were made over a period of 1 hour. Endotoxin was injected and continuous observations were carried out until the animals expired, or for at least 7 hours.

Exp. 2. The purpose of this experiment was to determine if pre-treatment of dogs with EACA would modify the severity of endotoxin shock. Ten dogs were anesthetized and control observations were made for 30 to 60 minutes, following which each of the dogs was given an infusion of 2 g of EACA over a period of 1 hour. During this time further measurements were made. At the end of the hour, endotoxin was injected, and the animals were continuously observed for a minimum of 7 hours.

Exp. 3. In this experiment the object was to determine if the severity of shock could be modified by giving EACA after injection of endotoxin. A group of 7 anesthetized animals were observed for a control period of 1 hour and then they received a lethal dose of endotoxin. Within 15 to 30 minutes after injection of endotoxin a sufficient amount of EACA was infused so that the blood pressure was stabilized, and a flow of urine occurred.

Exp. 4. Further to evaluate the post-endotoxin effect of EACA, 7 anesthetized animals were infused with a maximum of 2 g of EACA 40 to 80 minutes after receiving endotoxin.

Results. Control animals. Data on the 12 control dogs are shown in Table I. Mean survival time after injection of endotoxin was 12.6 hours. It will be noted that there was a precipitous drop in blood pressure in all of the animals, except 1 (Dog #9), within 15 minutes after injection of endotoxin. Over the succeeding hours a progressive hypotension developed, which was accompanied by oliguria and anuria. In addition (Fig. 1), the animals manifested an increase in hematocrit and a decline in blood pH.

Animals pre-treated with EACA. In a

* We are indebted to Dr. Sol Sherry, Washington Univ. Med. School, St. Louis, for the initial supply of EACA.

† Supplied by Merck Sharp and Dohme Research Laboratories, West Point, Pa.

TABLE I. Systolic Blood Pressure (BP) and Urine Output in cc (UO) in 12 Male Dogs Given a Lethal Dose of *E. coli* Endotoxin. Determinations recorded for a control period of 1 hr, then hourly after endotoxin.

Time of observation	Dog No. and wt (kg) 1 11.5 BP	UO	2 8.8 BP	UO	3 12.0 BP	UO	4 9.4 BP	UO	5 8.8 BP	UO	6 7.7 BP	UO
Control	150	8.8	140	9.2	115	10.9	150	10.0	215	9.9	145	2.0
15 min. post-endo.	40		35		30		25		40		50	
1 hr *idem*	40	12.4	30	11.1	50	0	80	18.6	40	12.0	50	0
2 " "	0	.9	0	0	50	0	75	2.3	0	0	45	0
3 " "					0	0	75	4.0			35	0
4 " "							85	5.0			0	0
5 " "							100	5.1				
6 " "							120	.6				
7 " "							70	.3				
Hr of survival post-endotoxin	2		2		3.5		18		2.5		4.0	

Time of observation	Dog No. and wt (kg) 7 13.8 BP	UO	8 8.8 BP	UO	9 11.0 BP	UO	10 8.0 BP	UO	11 7.0 BP	UO	12 11.0 BP	UO	Mean hourly value (dogs 1-12) BP	UO
Control	175	8.8	200	9.1	135	7.2	155	15.6	140	10.2	155	7.2	156	9.0
15 min. post-endo.	50		35		125		40		60		45		48	
1 hr *idem*	110	2.5	120	1.6	80	2.3	100	.5	110	11.4	65	8.1	73	6.7
2 " "	110	2.6	70	.3	110	2.4	50	.2	115	.8	65	.4	58	.8
3 " "	120	1.1	125	.5	135	4.8	70	.7	120	.4	110	1.1	66	1.2
4 " "	135	7.3	120	.4	135	4.8	0	0	120	.6	130	1.4	60	1.6
5 " "	110	14.8	0		135	4.1			120	.4	135	2.0	50	2.2
6 " "	110	14.0			125	4.3			120	0	120	.6	50	1.7
7 " "	110	14.6			120	5.0			80	0	105	0	40	1.7
Hr of survival post-endotoxin	56		5		18		4		12		13			

preliminary experiment rapid administration of 2 g of EACA resulted in a marked rise in blood pressure. For this reason, the EACA was slowly infused over a period of 1 hour. However, (Table II and Fig. 1), some hypertensive effect was still observed. The hypertension was not due to volume of fluid injected. Compared with the control group of animals the blood pressure became stabilized after injection of endotoxin. Seven of the 10 dogs survived. Severe oliguria and anuria did not occur in the majority of treated animals, and hemoconcentration and acidosis were minimal (Fig. 1).

Animals given EACA 15 to 30 minutes after endotoxin. A group of 7 dogs given a lethal dose of endotoxin manifested the initial decline in blood pressure, then within 15 to 30 minutes EACA was administered until the blood pressure became stabilized near pre-endotoxin levels. All 7 animals survived. One outstanding feature was the excellent output of urine manifested by all of the dogs (Table III) Amounts of EACA employed varied between 0.2 to 2 g, with an average of 1.2 g infused 25 minutes after endotoxin.

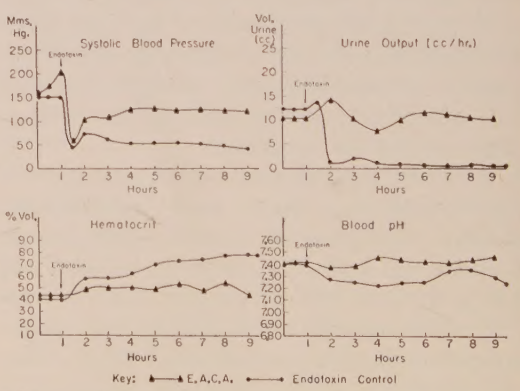

FIG. 1. Comparative mean hourly determinations of systolic blood pressure, urine output, hematocrit and blood pH in 10 control dogs given a lethal dose of endotoxin, and 10 dogs pre-treated with EACA. All of the control dogs died, while 7 out of the 10 treated animals survived.

TABLE II. Systolic Blood Pressure (BP) and Urine Output in cc (UO) in 10 Male Dogs Pretreated with 2 g Epsilon-Aminocaproic Acid and then Given Lethal Dose of *E. coli* Endotoxin. Determinations recorded for control period of 1 hr, 1 hr after EACA, then hourly after endotoxin.

Time of observation	Dog No. and wt (kg) 1 9.5		2 12.0		3 12.7		4 10.7		5 9.0		6 11.0		7 12.4		8 11.6		9 9.1		10 12.0		Mean hourly value	
	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO
Control	150	10.6	130	9.8	140	8.8	150	11.2	145	7.2	175	12.4	215	8.1	45	4.2	180	11.1	165	8.5	150	9.2
1 hr post-EACA	285	11.0	175	10.4	155	9.6	160	10.1	205	7.0	185	13.9	290	10.0	155	5.4	265	10.8	230	9.5	211	9.8
15 min. post-endo.	70	—	60	—	35	—	50	—	45	—	130	—	36	—	90	—	45	—	45	—	61	—
1 hr *idem*	130	18.0	150	32.7	65	6.9	75	6.0	60	13.6	155	37.4	60	4.1	118	4.2	70	15.0	175	4.6	106	14.3
2 " "	90	00 0	125	13.4	80	12.6	95	5.9	70	.1	160	65.4	135	0	87	.2	100	6.5	130	2.5	107	10.7
3 " "	145	1.2	135	11.3	100	8.6	125	14.9	120	2.3	150	24.5	130	0	100	.2	125	.6	110	4.2	124	6.8
4 " "	125	.2	145	27.4	100	22.3	140	21.2	125	9.6	150	13.0	100	0	125	0	145	7.0	95	3.6	125	10.4
5 " "	110	.2	145	20.3	85	23.2	140	24.0	120	18.0	135	16.5	0	0	100	0	150	26.0	115	1.2	122	12.9
6 " "	110	.3	145	19.4	85	10.1	140	23.6	115	12.4	140	14.3	—	0	100	.4	150	28.0	120	3.6	123	11.2
7 " "	105	.4	140	18.5	85	0	135	22.1	120	6.8	135	15.8			95	.6	145	38.4	115	4.8	119	10.7
Outcome	Survived		Survived		Died 7 hr		Survived		Survived		Survived		Died 4.5 hr		Survived		Survived		Died 18 hr			

TABLE III. Systolic Blood Pressure (BP) and Urine Output in cc (UO) in 7 Male Dogs Given Lethal Dose of Endotoxin, then Treated with Epsilon-Aminocaproic Acid 15 to 30 Min. after Endotoxin. Measurements recorded for control period of 1 hr, then hourly after endotoxin.

Time of observation	Dog No. and wt (kg) 1 11.4		2 11.2		3 9.0		4 14.0		5 10.2		6 12.0		7 9.0		Mean hourly value	
	Dose of EACA (g) and min. given after endotoxin 1.0–20		0.2–30		1.2–15		1.0–15		2.0–15		1.6–22		0.8–25			
	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO
Control	210	8.8	135	6.4	175	10.8	195	9.6	140	10.8	210	7.8	170	8.0	176	8.9
15 min. post-endo.	45		25		45		50		45		50		65		46	
1 hr *idem*	225	26.4	105	5.7	60	8.7	185	50.0	110	8.0	100	6.8	85	4.1	124	15.6
2 " "	180	46.2	165	20.2	90	.5	90	8.2	140	14.6	105	8.0	130	2.0	129	14.2
3 " "	210	40.6	145	.7	105	3.2	125	1.6	145	12.1	155	11.9	140	4.5	146	10.6
4 " "	165	76.0	175	1.6	115	9.8	160	7.0	160	9.5	150	12.3	165	11.5	155	18.0
5 " "	145	45.0	180	5.0	105	9.6	170	4.6	150	16.0	160	11.0	170	14.0	154	15.0
6 " "	165	46.0	175	6.8	105	32.0	160	8.2	140	18.0	150	10.1	160	15.8	150	19.5
7 " "	150	50.0	170	8.9	100	63.0	170	7.3	140	19.5	140	9.0	150	18.8	145	25.2

TABLE IV. Systolic Blood Pressure (BP) and Urine Output in cc (UO) in 7 Male Dogs Given Lethal Dose of Endotoxin, then Treated with 2 g Epsilon-Aminocaproic Acid 45 to 80 Min. after Endotoxin. Measurements recorded for control period of 1 hr, then hourly after endotoxin.

	Dog No. and wt (kg)																	
	1		2		3		4		5		6		7					
	11.0		10.0		10.5		7.3		8.6		5.9		6.1					
	Min. after endotoxin EACA given																Mean hourly value	
Time of observation	60		45		60		70		75		80		50					
	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO	BP	UO			BP	UO
Control	145	5.9	170	9.5	135	5.8	145	8.8	200	9.4	140	9.6	155	8.8			156	8.3
15 min. post-endo.	60		50		45		40		30		30		45				43	
1 hr *idem*	55	.2	65	2.8	45	2.0	30	20.0	175	3.2	135	5.7	135	4.9			91	3.0
2 " "	60	0	50	0	70	0	55	.8	170	4.8	170	30.9	140	11.8			102	6.9
3 " "		0			80	3.0	50	0	145	.9	143	11.2	190	14.0			87	4.2
4 " "					85	3.0	0		150	.4	145	4.9	185	15.0			81	3.3
5 " "					80	3.0			150	1.2	167	11.2	170	8.0			81	3.3
6 " "					75	3.0			145	.9	165	5.5	170	2.1			79	1.6
7 " "					70	2.8			140	1.0	160	3.0	165	0			76	1.0

Animals given EACA 45 to 80 minutes after endotoxin. In the preceding experiment dogs were given EACA during the initial phase of shock. In the present experiment 2 g of EACA were administered to a group of 7 dogs when the animals were in a more advanced stage of vascular collapse, but at a period when the shock could be reversed by other means, such as by a combination of a steroid and pressor agent(15). None of the 7 dogs survived. EACA given at the later stage of collapse did not correct the hypotension and oliguria (Table IV).

Discussion. These experiments have demonstrated that EACA, an inhibitor of plasminogen activation, protected dogs against lethal endotoxin shock, when EACA was infused either before or shortly after injection of endotoxin. This EACA-endotoxin time relationship is of interest in connection with observations that Sherry and his group(12) had carried out on the pyrogenic effect of endotoxin in human subjects who were given EACA. A hyperfibrinolytic state was induced with endotoxin (Pyrexal), then 1 hour after injection of endotoxin 10 g of EACA was administered. Although fibrinolysis was appreciatively decreased by this procedure, the endotoxin provoked the characteristic chills and fever. But no information is available as to whether pre-treatment with EACA would eliminate or modify the pyrogenic effect of endotoxin.

During these experiments, 2 consistent pressor changes occurred in dogs receiving EACA that are not readily explained. First, the infusion of EACA caused a significant rise in systemic blood pressure in normal anesthetized animals, particularly when concentrated solutions were used. The hypertension was detected because the pressure was monitored continuously. This phenomenon has not been reported by others. The pressor response was observed with 3 different sterile lots of EACA. Second, although EACA did have a pressor effect, pre-treatment of the dogs with the compound did not prevent, or apparently modify, the immediate hypotensive action of endotoxin that is unique for the dog. This initial hypotension, occurring within seconds after injection

of endotoxin, is due to intrahepatic venous constriction, which results in pooling of blood within the liver and walls of the intestinal tract. It is generally accepted that the vasoconstriction is due to histamine.

While it can be postulated that endotoxin activates the plasminogen-plasmin system with an increase in fibrinolytic activity and liberation of histamine, our experimental data are inadequate for the support of such a concept. It is possible that endotoxin activates proteolytic enzyme activity that is distinct from the plasminogen system. The foregoing results indicate that further information along several lines is needed. Quantitative plasminogen studies should be carried out in control animals, and in those pretreated with EACA, and quantitative histamine determinations should be made on the hepatic venous blood in similar groups of animals. The dose of both endotoxin and EACA should be varied in several groups of dogs.

If endotoxin does not activate the plasminogen-plasmin system in rabbits, as reported by others(10), it would be desirable to determine if pre-treatment of rabbits with EACA would protect them against a single lethal dose of endotoxin. Preliminary experiments in this laboratory indicate that EACA does not significantly protect this species.

The present experiments emphasize the complex nature of endotoxin shock. Incorporation of the coagulation mechanism into a concept on the pathogenesis of this type of shock requires more quantitative data in different mammalian species. The accumulating evidence suggests that several mechanisms may be responsible for the hemodynamic alterations caused by endotoxin, and there are important variations between species.

Summary. 1. Lethal shock was established with a standardized dose of *E. coli* endotoxin in adult mongrel dogs. In this species initiation of the peripheral vascular collapse involves liberation of histamine, which may be related to the activation of a proteolytic system by the endotoxin. 2. Pre-treatment of dogs with epsilon-aminocaproic acid (EACA), a potent inhibitor of plasminogen activator, protected the majority of animals against a lethal dose of endotoxin. Protection was also observed when EACA was given up to 30 minutes after injection of endotoxin. However, when EACA was administered beyond this time no protection occurred, and there was no evidence that the severity of the shock was modified. It is emphasized that these observations with EACA apply only to the dog. 3. The primary purpose of the present experiments was to determine if EACA protected dogs against lethal amounts of endotoxin. The results do not permit the conclusion that EACA modified the severity of shock by blocking plasminogen activation, and in this manner prevented liberation of histamine. There remains the possibility EACA blocked proteolytic activity that is independent of the plasminogen-plasmin system.

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A Quantitative Approach to the Study of Inflammatory Cells.* (26300)

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The presence of antigenic material within an organism produces not only localized inflammatory responses but also changes in number and types of cells found in regional lymph nodes, spleen and hemopoietic tissues (1,2). Antibody is formed and the organism altered in such a manner that future reexposure to the same antigen produces different magnitudes of cellular and humoral responses (3,4). To achieve an understanding of the origin, function and fate of the cells involved in these responses, technics for enumeration of cells migrating into an inflammatory area, as well as those leaving the area, must be applied. This can be accomplished by inducing the inflammation in the peritoneal cavity where routine hematological technics can be applied to determine amount of free fluid and total number of each cell type suspended in the fluid(5,6). This paper outlines a procedure for quantitative estimation of cellular changes in the inflammatory exudate following intraperitoneal injection of an antigen into sensitized and non-sensitized mice.

Materials and methods. Eighty C57 BL/6 mice, 3 to 8 months of age, were used in these experiments. One half of the animals were sensitized (immunized) by a series of 7 subcutaneous injections of 5 Lf Diphtheria toxoid (Lederle). Thirty days after the last sensitizing injection, 30 mice from each group were injected intraperitoneally with 5 Lf of Diphtheria toxoid in 0.4 ml isotonic saline. The remaining mice were used as uninjected controls. Daily autopsies were performed on 3 animals from each injected group and on one from each of the uninjected groups.

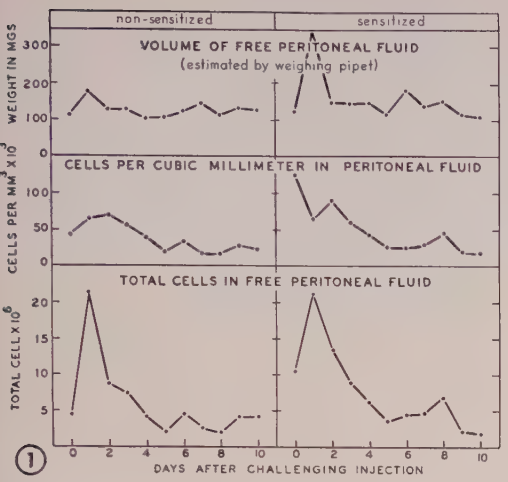
Sufficient fluid is usually present in the peritoneal cavity of mice to permit total cell counts and to make smears for differential counts. However, in the experiments reported here, an injection of 0.2 ml of fluid was made immediately prior to autopsy to assist in washing out the cells and to assure that a sufficient number of cells would be present to perform not only quantitative counts, but also to permit *in vitro* metabolic studies. The cellular responses obtained with diluted peritoneal fluid aspirate corresponded with data obtained in other experiments in which the peritoneal fluid was not diluted at time of autopsy.

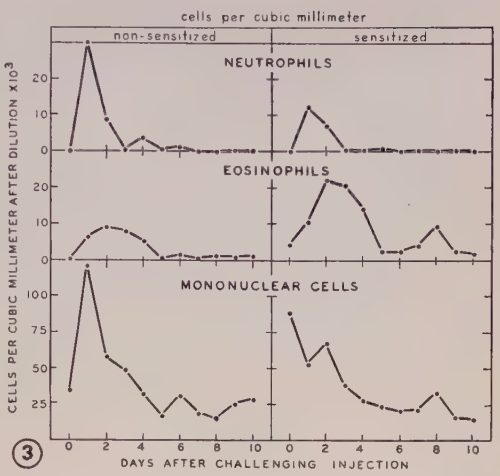
The autopsies were performed as follows: The animals were weighed and given an intraperitoneal injection of 0.2 ml of fluid consisting of equal parts of Hank's salt solution and polyvinylpyrrolidone (Schenley). They were then immediately killed by severing the cervical spinal cord. The inflammatory cells were aspirated from the peritoneal cavity, weighed, and samples taken for total counts, eosinophil chamber counts and for differential cell counts. Carpentier's solution† was used as a diluent since it enables both total cell and eosinophil cell counts to be performed. Counts were made in Speirs-Levy Eosinophil chambers, (an average of 370 cells per animal). The slides for differential counts were stained for 3½ minutes in May-Gruenwald Blood stain after methyl alcohol fixation. Differential cell counts were estimated from a minimum of 500 cells per experimental animal.

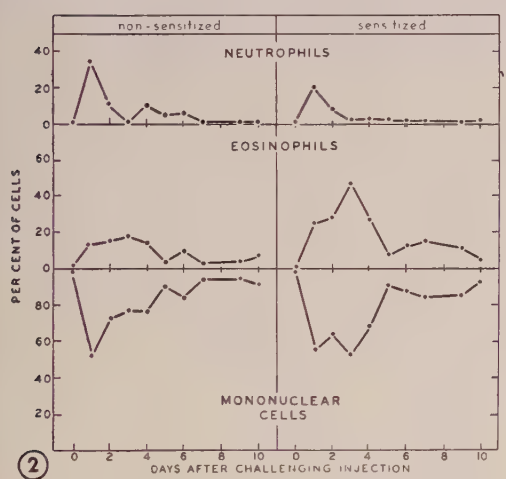
Results. Fluid and cell determinations obtained at various times after an intraperitoneal injection of diphtheria toxoid into sensitized and non-sensitized mice are averaged and illustrated in Fig. 1-4. The greatest amount of fluid in both groups was found in animals autopsied 24 hours following intraperitoneal injection, when the amount of fluid in the sensitized mice was approximately

* These data were presented at Federation Meetings, Chicago, Ill., April 1960. This work was supported by research grants from Nat. Inst. Health, P.H.S.

† Formula for Carpentier's solution(7): 1 ml eosin Y (2% aqueous solution), 3 ml neutralized formalin, 96 ml distilled water.







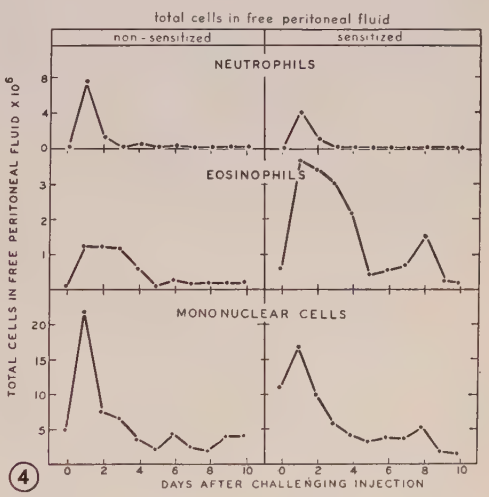


FIG. 1. Fluid volume and cellular changes in peritoneal exudate following intraper. inj. of diphtheria toxoid into sensitized and non-sensitized mice.

FIG. 2. Qualitative cellular response following intraper. inj. of diphtheria toxoid.

FIG. 3. Quantitative cellular response following intraper. inj. of diphtheria toxoid (cells per cu mm³).

FIG. 4. Quantitative cellular response following intraper. inj. of diphtheria toxoid (total numbers of each cell type which could be aspirated from peritoneal cavity).

twice that which could be obtained at other times (Fig. 1).

Number of cells per cubic millimeter of aspirated fluid is also shown in Fig. 1. Total cell estimate was obtained by multiplying number of cells per cubic millimeter by volume, assuming that 1 cmm was equivalent to 1 mg. The greatest number of cells which could be aspirated from the peritoneal cavity was found on the first day after antigen injection.

Fig. 2 illustrates the types of cells found in the peritoneal exudate. Fluid taken from the non-inflamed peritoneal cavity (0 day) consisted almost entirely of mononuclear cells (96%), the remainder being eosinophils, neutrophils and mast cells. In earlier experiments the mast cells did not show significant changes in number and they were not recorded at this time(4,5,6).

Injections of diphtheria toxoid produced rapid changes in cellular population of the peritoneal cavity which tended to persist throughout the 10-day period.[3] At 24 hours,

number of neutrophils had increased from less than 1% to 34% in the non-immunized mice and to 20% in the immunized mice. During the first 7 days both percent and total number of neutrophils were higher in the non-immunized animals than in the immunized mice. On the other hand, the eosinophil response was consistently higher in the immunized mice. Within 24 hours after antigen injection the number of eosinophils which could be removed from the peritoneal cavity of immunized mice increased from 58,000 to 3.6 million. By the third day, the eosinophils comprised 47% of all the cells found in the peritoneal cavity of the immunized animals. See Fig. 3.

The number of mononuclear cells in the peritoneal aspirate followed the same curve in both injected groups (Fig. 4). The maximum number of cells was found 24 hours after antigen injection, followed by a marked decrease between the 1st and 3rd days. Phagocytized material was commonly observed in the mononuclear cells. During the first day numerous vacuoles containing necrotic neutrophils as well as antigenic material were seen. Between the 3rd and 8th days, eosinophils in various stages of degeneration were noted within the macrophages of the sensitized animals. Morphological differences in the mononuclear cells were also apparent, beginning on 4th day of inflammation. Cells with intense cytoplasmic basophilia as well as plasma cells were seen, particularly in the sensitized animals. However, since transitional forms made clear-cut classification difficult, these cells were all grouped as mononuclear cells.

Discussion. The results presented here indicate that the cells taking part in an inflammatory response can be conveniently studied in the peritoneal cavity. Routine hematological technics can be applied at various times after initiation of the inflammation and a sufficient number of cells removed to study both qualitative and quantitative changes.

Comparisons were made of different methods of enumerating the inflammatory cells. Qualitative data, expressed as percent (Fig. 2) can be compared with quantitative data, expressed as cells per cmm (Fig. 3) or total cells in the aspirate (Fig. 4). The neutrophil counts appear similar regardless of the method used for presenting the data. On the other hand, comparison of the eosinophil counts indicates marked differences. The increase in fluid volume, as well as the numerical increase in other inflammatory cells, tends to mask the eosinophil response when qualitative data alone are considered.

Mononuclear cells make up a heterogeneous group comprising approximately 96% of all cells normally found in the uninflamed peritoneal fluid. Following an injection of any inflammatory material, the granulocytes migrate into the peritoneal cavity and therefore markedly decrease the proportion of these cells (Fig. 1). However, when the cells per cubic millimeter and the increased fluid volume are taken into account the mononuclear cells are found to greatly increase in number (Fig. 2,3) during the first 24 hours. This would be impossible to determine from the qualitative data alone (differential counts).

Although both granulocytes and mononuclear cells were present in maximum numbers on the first day after injection, there were distinct differences in rate of disappearance of each cell type on subsequent days. Neutrophils decreased in number very rapidly and were seldom seen after the 2nd day. On the other hand, eosinophils tended to persist for longer periods, especially in the immunized animals. The mononuclear cells decreased in number between the 1st and 3rd days but throughout the whole period remained the predominant cell type in the inflammatory exudate.

The factors involved in disappearance of inflammatory cells vary, depending upon the cell type. Neutrophils are phagocytized by mononuclear macrophages, and the remains of these cells were found in macrophages within 24 hours of onset of inflammation. Eosinophils are also phagocytized by macrophages, but usually between the 3rd and 8th day of inflammation. Although phagocytosis of injured mononuclear cells may occur, many of the cells appear to be viable and are carried out of the inflammatory area into the lymphatic and blood vessels by the ede-

metous fluids(2). Thus, rate of phagocytosis of the granulocytes, as well as rate of migration of the mononuclear cells from the inflammatory area, account to a great extent for the sequence of cell types seen in the inflammatory exudate.

The number of each cell type responding to an injection of antigen varies depending, in part, upon sensitivity of the animal to that antigen. The neutrophil response appears to be somewhat greater in animals injected with antigen for the first time than in reinjected animals. In contrast to neutrophils, eosinophils are found in greater numbers when the antigen is reinjected. The local increase in eosinophils is a consistent finding whenever an antigen is repeatedly injected into an animal(4). This has recently been confirmed by Litt(11) who observed a quantitative local eosinophilia in the guinea pig following repeated intraperitoneal injections of an antigen. Archer(12) has also found local increases in eosinophils at the site of reinjection of antigen in the skin of the horse. The relation of these cells to adrenal cortical hormone secretions and to antibody formation has been presented earlier(8,9,10).

The mononuclear cells of the sensitized animals showed marked changes in morphological appearance during various periods of the inflammation. Intense basophilic staining of the cytoplasm was observed between the 4th and 10th days of inflammation. It is considered significant that these changes occur in macrophages which have engulfed eosinophils(2,3).

Summary. This paper compares procedures for qualitatively and quantitatively studying cellular responses to inflammatory substances. It was noted that estimates of total cells in the aspirated exudate suggested conclusions not obvious from data obtained from differential counts. These data suggest that the observed sequence of inflammatory cells is due not only to the initial migration of cells into the inflamed area, but to the rate of phagocytosis of the granulocytes by the macrophages. The inflammatory exudate of sensitized animals contained fewer neutrophils and greater numbers of eosinophils. Plasma cells and mononuclear cells with intense basophilic cytoplasm were frequently observed following the disappearance of eosinophils.

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DNA Synthesis in Inflammatory Cells of Immunized and Non-Immunized Mice.* (26301)

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This study utilizes quantitative technics developed earlier(1) to determine the number of cells synthesizing DNA in the inflam-

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matory exudate of immunized and non-immunized mice. Recent experiments indicate that thymidine incorporation occurs during interphase in cells which are doubling their nuclear DNA preparatory to mitosis(2,3,4, 5). Cells during various stages of inflammation were incubated *in vitro* with tritiated thymidine, and incorporation of radioactivity into nuclei was determined by autoradiography.

Materials and methods. A total of 80 adult C57 BL/6 mice were used in these experiments. One-half of the animals were sensitized (immunized) by a series of 7 subcutaneous injections of 5 Lf Diphtheria toxoid (Lederle). Thirty days after the last sensitizing injections, 30 mice from each group were injected intraperitoneally with 5 Lf of the toxoid in 0.4 ml isotonic saline. The remaining mice were used as uninjected controls. Each day, autopsies were performed on 3 animals from each injected group, and one animal from each uninjected group. Data were omitted for "day 8" because of technical difficulties in preparation of the autoradiographs.

At time of autopsy, 0.2 ml of a suspending fluid, consisting of polyvinylpyrrolidone (Schenley) and Hank's balanced salt solution, was injected intraperitoneally. The inflammatory exudate was immediately removed by aspiration, samples were taken for counting, and the remaining cell suspension (approximately 0.20 ml) added to 0.05 ml pyrogen-free saline containing 1 μc tritiated thymidine (360 μc/mM Schwarz). The cells were incubated for 1 hour, cooled to 5°C, then painted on gelatine-subbed glass slides, using the brush technic of Burke, *et al.* (6). The slides were then air dried, fixed in methyl alcohol, washed for 15 minutes in distilled water, and dipped into NTB^3 liquid nuclear emulsion (Kodak) according to procedures outlined(7,8). After exposure for 7 days at 5°C, the slides were developed in D-19 for 2 minutes, fixed, washed and stained for 3½ minutes in May-Gruenwald Blood stain.

The proportion of inflammatory cells incorporating tritiated thymidine was estimated by counting approximately 5,000 cells on the autoradiograms of each animal. Only cells containing 6 or more silver grains over the nucleus were recorded as labeled cells. Number and types of cells at each stage of inflammation were calculated(1) and total number of cells incorporating thymidine estimated.

Results. Examination of the autoradiograms indicated that only mononuclear cells were capable of *in vitro* incorporation of the tritiated thymidine. In no case was a label obtained over neutrophils, eosinophils or mast cells. The number of mononuclear cells capable of incorporating thymidine in the uninjected (0 day) animals averaged 36/10,000 (0.36%) cells in the normal, and 9/10,000 (0.09%) cells in the immunized animals. (Table I and Fig. 1 Top).

In the non-immunized animals injected with diphtheria toxoid, a total of 839 labeled cells were found among 130,000 inflammatory cells. Average number of labeled cells for the period from 1 to 10 days was 64/10,000. A gradual increase in proportion of cells incorporating thymidine was observed, beginning on the 3rd day, and reaching a peak on the 7th day (181/10,000).

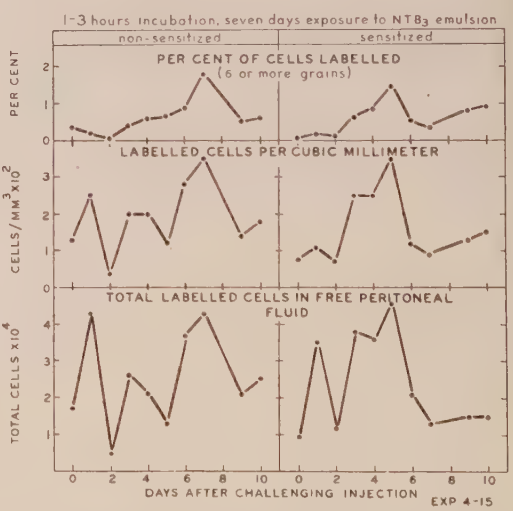

FIG. 1. *In vitro* incorporation of tritiated thymidine in inflammatory mononuclear cells of non-sensitized and sensitized mice. Calculations were made of No. of labeled cells per cmm, and total No. which could be aspirated in the inflammatory exudate. Labeling was obtained by incubating the cells with thymidine, and exposing them to NTB_3 emulsion, for 7 days.

TABLE I. *In Vitro* Incorporation of Tritiated Thymidine by Mononuclear Cells in an Inflammatory Exudate.

		Days after intraper. inj.									
	Animal	0	1	2	3	4	5	6	7	9	10
Total mononuclear cells in aspirate ($\times 10^6$)	Normal	4.8	21.7	7.4	6.3	3.5	2.0	4.3	2.4	4.0	4.0
	Sens.	11.0	17.7	9.9	5.8	4.0	3.2	3.8	3.6	1.8	1.6
Labeled cells per 10^4 mononuclear cells	Normal	36	20	6	40	64	68	87	181	52	78
	Sens.	9	20	11	65	87	145	65	37	83	90
Total No. of labeled cells in aspirate ($\times 10^3$)	Normal	17.1	43.0	4.5	26.0	21.0	13.0	37.0	43.0	21.0	25.0
	Sens.	9.5	35.4	12.0	38.0	36.0	46.0	21.0	13.0	15.0	15.0

In the immunized animals a total of 1170 labeled cells were found among approximately 156,000 cells. Average number of labeled cells for the period from 1 to 10 days was 67/10,000. A gradual increase in proportion of labeled cells also occurred beginning on the 3rd day, and reaching a peak on the 5th day (145/10,000).

Calculations were also made of number of labeled cells per cubic millimeter, and total number which could be aspirated from the peritoneal cavity (Table I, Fig. 1). An in-

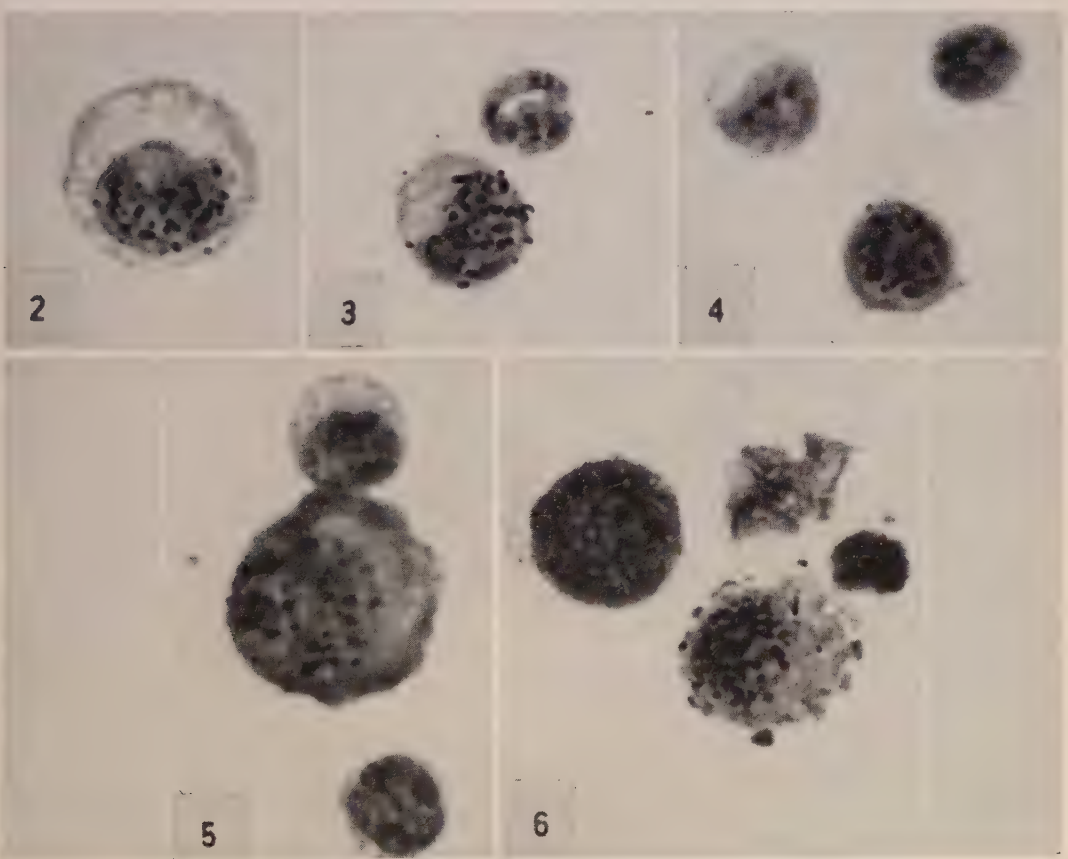

FIG. 2-6. Inflammatory cells incorporating tritiated thymidine *in vitro*.

FIG. 2. Histiocytes. Cells which most frequently incorporate thymidine.
FIG. 3. Histiocyte and an unlabeled eosinophil.
FIG. 4. Medium-sized lymphocyte.
FIG. 5 & 6. Basophilic transitional cells which appeared between 4 and 7 days in immunized group but were occasionally seen in non-immunized animals.

crease in actual number of cells capable of synthesizing thymidine occurred during the first day of inflammation in both groups. This increase was not evident when percentage data alone were considered.

The types of labeled mononuclear cells varied, depending upon stage of inflammation. The majority of labeled cells at all stages consisted of large macrophages or histiocytes (Fig. 2,3), and medium sized lymphoid cells (Fig. 4). Beginning on the 4th day, cells with varying amounts of cytoplasmic basophilia became labeled (Fig. 5 and 6). In immunized mice these basophilic cells averaged 13% of all the labeled cells found between 4th and 10th days. In the non-immunized mice approximately 2.5% of the labeled cells were basophilic during this same period.

All stages of transition between macrophages and plasma cells were observed, and it was impractical to classify the cells into distinct morphological groups.

Discussion. These experiments demonstrate a procedure for study of metabolic activity of inflammatory cells. A comparison was made of number of cells capable of *in vitro* incorporation of thymidine in the inflammatory exudates of immunized and non-immunized mice injected with Diphtheria toxoid. The cells which were found to incorporate thymidine are mononuclear cells—a heterogeneous group including lymphoid cells, histiocytes, macrophages and cells with varying amounts of basophilic-staining RNA in their cytoplasm. The granulocytes found in the inflammatory exudate are end cells which, since they are incapable of cell division, do not incorporate thymidine.

The number of inflammatory cells capable of incorporating tritiated thymidine was small compared with the number of such cells found in bone marrow or spleen. During the first 2 days of inflammation, total number of peritoneal exudate cells incorporating thymidine never exceeded 46,000, although, within the first 24 hours, there was an increase of more than 10 million mononuclear cells in the exudate of the non-sensitized animals. These additional cells must arise by migration of mononuclear cells into the exudate, since the DNA synthesizing cells were too few in number to account for the increase. These data do not indicate whether the migrating cells originate in blood vessels, omentum, mesenteries or adjoining tissues. Other experiments, however, have demonstrated that at least some of the mononuclear cells must migrate from the blood vessels along with the granulocytes(9,10,11).

Following the first day of inflammation, there is a disappearance of mononuclear cells due, in part, to necrosis and disintegration of the cells, and, in part, to migration of the cells out of the inflammatory area and into the lymphatic and blood vessels(1,12). There is, thus, a large turn-over of cells moving both in and out of an inflammatory exudate, and a true picture of this turn-over can be obtained only by considering quantitative data.

Greater numbers of mononuclear cells with intense basophilic cytoplasm (due to RNA synthesis) were observed in the sensitized animals(12). These cells also appear to incorporate amino acids(13) and are probably involved in the synthesis of antibody(12,14, 15). Although differences in morphological appearance were apparent in the sensitized mice, total number of cells present, and number synthesizing DNA, were identical to that of the non-sensitized animals. The sensitized animals appeared to synthesize DNA somewhat sooner than the non-sensitized animals, possibly because they are more efficient in rate of phagocytosis and removal of antigen (16). There was no indication that these cells were dividing to form plasma cells. Evidence available in our laboratory, and from others(17,18), does not support the concept that antibody-producing cells arise by mitosis, but rather suggests that there is a morphological transformation of macrophages, or similar mononuclear cells.

Additional experiments are in progress in which cells labeled with tritiated thymidine are traced from the inflammatory exudate into various organs and tissues of the body.

Summary. Inflammatory cells aspirated from the peritoneal cavity of mice injected with Diphtheria toxoid were studied by autoradiography for *in vitro* incorporation of

tritiated thymidine. The proportion of labeled cells was low prior to and for the first 2 days following initiation of inflammation. Beginning on the 3rd day the proportion of labeled cells gradually increased reaching a maximum on or about the 7th day in the non-sensitized mice. In animals previously sensitized to the Diphtheria toxoid, the increase in labeled cells occurred more rapidly, reaching a maximum on or about 5th day of inflammation. However, number of cells capable of DNA synthesis in both groups appeared to be identical. The origin of inflammatory mononuclear cells was discussed. Only a small proportion of the cells originate by division of cells suspended in the inflammatory exudate. The majority of cells, both granulocytes and mononuclear cells, migrate into the inflammatory area from the blood vessels and possibly from adjoining tissues.

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In vitro Histamine Release from Rat Mast Cells by Chemical and Physical Agents.* (26302)

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A variety of simple chemical compounds capable of *in vivo* and *in vitro* histamine release from cells has been investigated during the last 13 years(1). Most studies, particularly with compound 48/80, have utilized disruption and degranulation of tissue mast cells as an index of histamine release(1-6). Observations on mast cell degranulation may not be adequate for quantitative study of 48/80 and histamine release; indeed, histamine release may occur without degranulation(7). Furthermore, there is almost no quantitative information concerning other chemical releasers. Accordingly, we made direct measurements of histamine released after addition of various concentrations of compound 48/80 to rat peritoneal mast cells, and compared these effects with those of sev-

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† Student Summer Research Fellow, Allergy Foundation of America, 1958, 1959.

eral other substances including 1935L, a substituted butylamine(8).

Method and materials. A. *Source of mast cells.* The method of Padawer and Gordon, modified slightly, was used to obtain suspensions of intact rat peritoneal mast cells(9). Sixteen ml of a modified Tyrode's solution (no glucose) was warmed to 37°C and injected into the peritoneal cavity of male Sprague-Dawley rats weighing from 250 to 400 g. The abdomen of each animal was gently massaged for about one minute after injection. The animal was then exsanguinated rapidly by decapitation. The abdomen was opened and 10 to 12 ml of fluid withdrawn into a 20 ml siliconed‡ syringe with an 18 gauge needle. A 10 ml aliquot was added immediately to 20 ml of modified Tyrode's solution in a siliconed beaker kept at 37°C. The resulting 30 ml suspension of mast cells was termed the reference cell suspension, and will be referred to as such below. We found that the mast cell suspensions could be handled easily without addition of any anticoagulant.

B. *Determination of mast cell concentration.* Mast cells were counted by mixing one part of 0.1% dimethyltoluthionine chloride (toluidine blue) with 19 parts of the reference cell suspension in a standard white-cell counting pipette. The mixture was shaken vigorously and total number of nucleated cells was then counted in a hemocytometer with a counting chamber depth of 0.2 mm. All cells staining metachromatically with toluidine blue were assumed to be mast cells. These were easily recognizable because of their large size and typical intracellular granules.

C. *Release of histamine by compound 48/80 and other substances.* Two ml aliquots were taken from the reference cell suspension and placed in separate 12 ml siliconed centrifuge tubes. After the samples were warmed to 37°C various amounts of 48/80‖ in 0.05 ml volumes were added to different aliquots to provide the final concentrations indicated in *Table I.* The solutions were mixed by gentle tapping of each tube 10-12 times. Each animal provided a volume of reference suspension adequate for at least 6 determinations (each one being run in duplicate).

After 45 minutes incubation at 37°C and gentle agitation every 10 minutes, the suspensions were centrifuged at 350 g for 15 minutes to separate the cells. One ml of each supernatant solution was mixed with an equal volume of 10% trichloroacetic acid (TCA) and allowed to stand for 20 minutes at room temperature. The samples were then centrifuged at 1100 g for 8 minutes and one ml of the resulting 5% TCA supernatant solution used for histamine analysis.

Other agents, 1935L,¶ 4-4′-diamidinostilbene isethionate (Stilbamidine), protamine sulphate, and n-octylamine were likewise introduced in 0.05 ml volumes to give the concentrations shown in Table I. Other substances were tested at the single concentration indicated in Table II. The effects on pH of each concenrtation of each substance were also noted. D. *Effect of mast cell dilution on release of histamine by 48/80.* The reference cell suspension was diluted with an equal volume of modified Tyrode's solution and aliquots were subjected to 3 low concentrations of 48/80. The fraction of histamine released was compared with that produced from undiluted suspensions by these concentrations. E. *Effects of physical changes on release of histamine by compound 48/80.* Different aliquots of the reference cell suspension were brought to 4°, 20°, and 40°C. After 15 minutes, 0.05 ml of 48/80 was added to each (final concentration 10^{-6}M) and the same temperature maintained. After 20 minutes all samples were centrifuged at 6°C to separate the cells and the supernatant solutions were prepared for histamine assay as before. Investigation of osmotic effects was accomplished by diluting aliquots of reference suspension with one and 2 volumes of distilled water and measuring extracellular

‡ All glassware used in contact with mast cells was freshly siliconed with Desicote® obtained from Beckman Instrument Co., Fullerton, Calif.

‖ Obtained through the generosity of the late Dr. J. S. deBeer, Burroughs, Wellcome and Co., Tuckahoe, N. Y.

¶ Kindly supplied by Dr. B. N. Halpern, Hosp. Broussais, Paris, France.

TABLE I. The Chemical Releasers of Histamine.

Molar conc. of releaser	48/80 N*	48/80 Mean† ± S.D.	1935L N	1935L Mean ± S.D.	Protamine N	Protamine Mean ± S.D.	Stilbamidine N	Stilbamidine Mean ± S.D.	Octylamine N	Octylamine Mean ± S.D.
10^{-7}	5	.05 ± .02			2	.15				
2×10^{-7}	10	.32 ± .12	2	.01	3	.29 ± .19				
4×10^{-7}	8	.58 ± .07	2	.07						
10^{-6}	28	.76 ± .10			3	.66 ± .05	1	.07		
2×10^{-6}	3	.82 ± .12	4	.48 ± .07			2	.05		
5×10^{-6}			8	.49 ± .15	3	.74 ± .07				
10^{-5}	8	.92 ± .10	4	.55 ± .09	3	.88 ± .03	3	.16 ± .07	1	.08 pH 8.28
2×10^{-5}			4	.70 ± .07	2	1.0				
5×10^{-5}	6	.94 ± .07					3	.45 ± .16	1	.08 pH 8.3
10^{-4}	4	1.0 ± .10	10	.73 ± .07			3	.58 ± .18		
2×10^{-4}	3	1.0 ± .10					3	.77 ± .06	1	.78 pH 8.4
5×10^{-4}			6	.78 ± .08			4	.85 ± .12	2	.80 pH 8.5
1.6×10^{-3}									3	1.07 ± .06 pH 8.9

* N = No. of duplicate determinations.

† Fraction of available histamine released.

histamine as before. F. *Control samples.* In conjunction with each study, duplicate 2 ml aliquots were taken from the reference cell suspension and carried through the various procedures without addition of releaser or inhibitor. Also, total available histamine (cellular and free) of the reference suspension used for each study was measured by adding 1 ml of 10% TCA to duplicate uncentrifuged 1 ml aliquots. These preparations were mixed and centrifuged as above and the supernatant solutions, being rich in histamine, were diluted 5-fold. One ml aliquots were then used for histamine analysis. G. *Measurement of histamine.* All of the final solutions (containing 5% TCA) from the above experiments were buffered with 1/10 volume of 4M sodium acetate and histamine content measured chemically by the method of Lowry *et al.*(10). Each group of samples was analyzed along with duplicate standard solutions containing 0.25 μg of histamine base. All solutions were prepared using triple glass-distilled water and all glassware was meticulously cleaned and rinsed with triple distilled water before use.

Results. A. *Cell counts and histamine values.* A differential count of the reference suspensions from 74 rats (mean weight of 312 g) yielded 48 ± 21 mast cells per cubic mm, 5.8% of the total nucleated cells. Red cells were rarely seen. These reference suspensions contained 1.38 ± 0.51 μg/ml of intracellular histamine and 0.06 ± 0.02 μg/ml of free histamine. Calculating histamine per mast cell for each animal separately, we obtained the value of 29 ± 10 μg of histamine/10^6 mast cells, assuming that the other nucleated cells contained no histamine(11). Because of the biological variation, all data on histamine released have been expressed in terms of the fraction of available cellular histamine released rather than in absolute values.

B. *Histamine releasers.* A significant amount of histamine was released by 2×10^{-7}M 48/80 (Table I). Histamine release increased greatly with 48/80 concentrations up to 10^{-6}M. The release of histamine by 48/80 was found to be independent of mast cell concentration; the proportion of histamine released from diluted cellular suspensions was identical to that from the standard reference suspension after incubation with 10^{-7}, 2×10^{-7} or 4×10^{-7}M 48/80.

Compound 1935L was also an effective histamine releaser but less potent than 48/80 (Table I). Concentrations of 4×10^{-7}M and below released no appreciable histamine. A small increment (to 10^{-6}M) resulted in a marked rise in amount of histamine released but higher concentrations did not release as much "available" histamine as 48/80.

Both protamine sulfate and n-octylamine

TABLE II. Other Releasers of Histamine.

Substance	Concentration	Release*
Parachloromercuribenzoate	10^{-3}M	.88
Formaldehyde	10^{-3}M	.40
5-hydroxytryptamine	3×10^{-5}M	.39
Phenol	10^{-2}M	.16
Tannic acid	10^{-2}M	.52
Ammonium oxalate	10^{-2}M	.31
Ethanol	35%	1.00
Methanol	50%	1.00

* Fraction of total available histamine released.

released more histamine than 1935L, but higher concentrations of stilbamidine were required and it exhibited a less abrupt rise in percentage of histamine in relation to increases in concentration. Other substances treated for their histamine releasing capacities are listed in Table II. Although we first used parachloromercuribenzoate as a potential inhibitor of histamine release, it released significant amounts of histamine at 10^{-3}M concentration so was not studied further. Both d-tubocurarine and toluidine blue produced high blank readings on histamine assay, so could not be studied.

The nature of our experiments required the pooling of data obtained by study of many animals; thus the ranges of values in Table I represent mainly the degree of biological variability. Large numbers of determinations were carried out at certain concentrations of releaser, particularly 48/80 (Table I). Most of these were control values for further studies concerning the inhibition of histamine release, to be reported later. The effects of pH change were not studied *per se*. None of the agents used altered the pH of the system significantly except for tannic acid (pH of 6.6) and octylamine (Table I).

As expected, several physical procedures caused release of histamine. Both freeze-thawing and heating to 62°C released cellular histamine completely. When an equal volume of distilled water was added to a mast cell suspension no histamine release occurred. However, addition of 2 volumes of water released 60% of the total histamine.

At 4°C, 10^{-6}M 48/80 released insignificant amounts of histamine. Histamine release by 48/80 occurred to about the same degree at 20°C, and 40°C as at the usual temperature of 37°C. However, as the temperature was increased above 40°C spontaneous release of histamine (control values) increased significantly.

Discussion. Our finding that 5.8% of the total number of peritoneal cells were mast cells is comparable to the 3.5% found by Padawer(12) and 5.2% by Lagunoff and Benditt(11). The histamine content, 29 μg/10^6 mast cells is surprisingly close to that reported by others(11,13).

Among the agents studied, compound 48/80 was the most effective histamine releaser on a molar basis; a concentration of 10^{-6}M released a majority of cellular histamine. Our observations with 48/80 are similar to those reported by Norton, who used degranulation of mast cells as an index of histamine release (4). Accordingly, histamine release and mast cell degranulation by 48/80 seem to be comparable quantitatively as well as qualitatively. There is little comparable quantitative information concerning the degranulation effects of the other agents studied.

Small concentrations of the more effective substances released a majority of available histamine, yet large increments were necessary to release the remainder. Indeed, large concentrations of 1935L and stilbamidine were not capable of releasing all "available" histamine. Some mast cells may have been more resistant to release than others; mast cells do not comprise a uniform population, varying in size, staining reactions, and sensitivity to drugs(4,5). Certain "mast" cells might be totally resistant to the effects of 1935L and stilbamidine. Octylamine was relatively weak, and a high concentration was required to produce total histamine release. Although the pH was high, it was not high enough to induce spontaneous histamine release(14,15). Mongar has demonstrated that only the non-ionized form of octylamine is active; this requires an alkaline pH, since the pKa of octylamine is 10.8(14). Thus, histamine release required a concentration of 2×10^{-4}M of total octylamine but the non-ionized form had a concentration of only 7.9×10^{-7} at pH of 8.4. Thus, this base is active in the same range as 48/80.

Protamine was also potent in terms of

molar concentration. However, this large polypeptide has a molecular weight of 5600; the concentrations used would be meaningful only if there is just one histamine-releasing site on each molecule.

The proportion of histamine released was dependent on the concentration of 48/80 but independent of the concentration of mast cells (and thus, the amount of available cellular histamine). The number of molecules of histamine released by one molecule of 48/80 thus would be proportional to the amount of cellular histamine. With 4×10^{-7}M 48/80, 58% of the intracellular histamine was released or about 0.8 μg of base (7.2×10^{-3} μmoles) from 1 ml of reference cell suspension. Thus, 1 molecule of 48/80 would release up to 18 molecules of histamine, and might have released more from more concentrated suspensions.

Summary. Release of histamine from suspensions of rat peritoneal mast cells under various conditions has been measured directly by a microchemical technic. Compound 48/80 released histamine in a concentration as low as 2×10^{-7}M and was the most potent substance investigated. Several other substances released histamine; the effects of compound 1935L, protamine, n-octylamine and stilbamidine were evaluated at several concentrations and compared with those of 48/80. Stilbamidine was considerably less potent than the others. As expected, certain physical changes released histamine, and low temperatures prevented 48/80 histamine release. Qualitative variations in response of these mast cells to different releasers suggested that mechanisms of histamine release vary considerably among the chemical agents now being investigated.

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Acute Effects of Guanethidine on Myocardial Contractility and Catecholamine Levels.*† (26303)

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Recent pharmacological reports describe guanethidine, [2-(octahydro-1-azocinyl)-ethyl]-guanidine sulfate, as a potent antihypertensive agent(1,2). An immediate period of adrenergic-like stimulation—manifested by hypertension, increased cardiac output, and positive chronotropism—exists after the agent is acutely administered to dogs(3). The objects of this study were to determine the initial changes in heart contractility and in plasma and tissue catechola-

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† Preliminary report in: *Symposium on Guanethidine*, Ciba Pharmaceutical Products, Inc., 1960, p75.

‡ Research fellow supported by Nat. Heart Inst.

mine concentrations produced by guanethidine.

Methods. Studies were done on acutely prepared, bilaterally vagotomized, open-chest dogs under pentobarbital anesthesia. Heart contractile force (isometric systolic tension) (IST) and arterial blood pressure were measured respectively with a strain gauge arch (4,5,6) and a Statham transducer. Strain analyzers and direct writing oscillographs were used to record these parameters.

Such studies were grouped in 4 series. In series 1, cardiovascular responses and plasma catecholamine levels were observed following acute injections of guanethidine§; in series 2, circulatory responses were similarly observed after various autonomic blocking effects had been produced; in series 3, tissue analyses of catecholamine content were made after administration of guanethidine for 2 days. In a 4th series with isolated rabbit hearts, inotropic and chronotropic responses to guanethidine were determined and the perfusates were analyzed for catecholamines following guanethidine.

In series 1, five animals were injected intravenously with guanethidine 5 mg/kg. Successive doses were given to 2 of these animals. Venous and arterial blood samples from the 3 other dogs were drawn during control and cardiovascular stimulation and analyzed for catecholamines(7). Three acutely prepared animals with intact vagi were administered guanethidine.

In series 2, dogs were treated as follows before receiving guanethidine: 2 received hexamethonium 10 mg/kg; epidural spaces of 2 dogs were irrigated with tetracaine 0.1% (8); the heart of one animal was denervated by extirpating the stellate through the fifth thoracic ganglia; 2 received phentolamine 0.5 mg/kg; 2 were atropinized 0.25 mg/kg; and 4 were injected intramuscularly with reserpine 0.1 mg/kg 48 and 24 hr prior to being prepared for IST and blood pressure recordings. In one animal the responsiveness of the adrenal glands to guanethidine and KCl(9) was compared by injecting each agent through a catheter into the thoracic aorta. Arterial blood samples withdrawn prior to and during cardiovascular stimulation after these intra-aortic injections were analyzed fluorimetrically for catecholamines(7).

In series 3, seven dogs administered guanethidine 5 mg/kg on two successive days were sacrificed on the third day with pentobarbital. Samples of left ventricular wall from all and aorta from 5 animals were analyzed fluorimetrically for catecholamines(10). An equal number of heart and aorta samples from 7 control dogs were analyzed similarly.

In series 4, ten isolated perfused rabbit hearts were injected with guanethidine 0.25-1.0 mg. Six hearts received successive doses. In addition 7 groups of 3 hearts were perfused. Perfusate samples were taken prior to and after injecting guanethidine 0.7 mg. Like samples from a group were pooled and analyzed for catecholamines(7). Isotonic contractions were recorded on a kymograph with a heart lever.

Results. Fig. 1 illustrates the typical cardiovascular response to guanethidine in

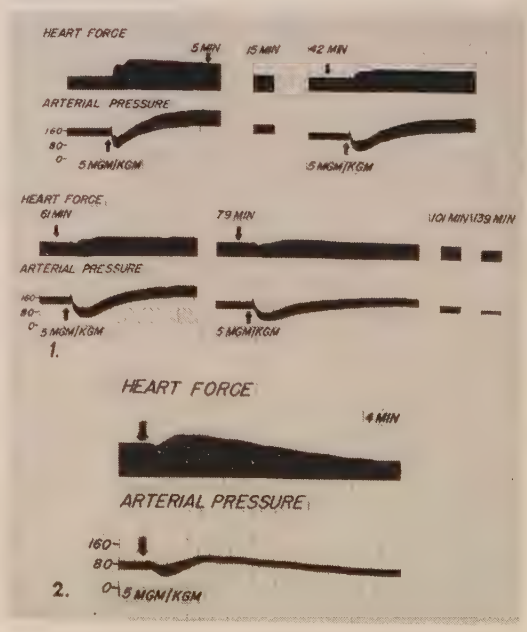

FIG. 1. Effects of repetitive doses of guanethidine on cardiovascular system.

FIG. 2. Effects of guanethidine after reserpine.*

§ Supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J., through the courtesy of Dr. Edgar A. Jack.

* 0.1 mg/kg reserpine I.M. 48 and 24 hr pre-exp.

TABLE I. Tissue Content of Catecholamines in Normal and Guanethidine Treated Dogs.

No.	Exp.	Tissue		Norepinephrine (μg/g)	Epinephrine (μg/g)
10	5	Aorta	Control	.89	.08
			After guan.*	.14 $P < .01$	.03 $P > .10$
14	7	Ventricle	Control	.51	.08
			After guan.*	.07 $P < .01$	.02 $P < .05$

* 5 mg/kg guanethidine IV 48 and 24 hr before analysis.

acutely prepared vagotomized dogs. With the 5 dogs of this series, hypotension averaging 34% (S.D. ± 7.9%) below control mean blood pressure persisted 48 sec (S.D. ± 5.9 sec). Mean blood pressure then rose to 85% (S.D. ± 24%) above control level. IST (heart force) increased abruptly. The initial positive inotropism decreased or reached a plateau before a more intense positive inotropism of longer duration developed. At most, 35 sec intervened between the two peaks. In 2 of 5 dogs, the primary and secondary peaks were fused. The latter peak averaged 121% (S.D. ± 36%) above the control. Blood pressure and inotropic responses persisted 40-60 min.

Of the animals repetitively injected, both displayed diminution in duration as well as in intensity of pressor and inotropic responses. Successive doses caused the hypotensive period to become more pronounced. Blood pressure leveled off below control after each dose. Following the fourth dose, IST was substantially below initial control.

Pressor and inotropic responses of animals with intact vagi were qualitatively similar but less intense and shorter in duration than those of vagotomized animals.

In dogs with preganglionic tetracaine blockade, pressor and inotropic responses elicited by guanethidine were qualitatively the same as in animals without blockade. The blockade, however, by removing sympathetic influences, reduced both pressure and IST to about half the previous values. Under these relatively depressed conditions, the percentage responses to guanethidine were considerably increased although maximal levels of recorded responses did not exceed those obtained under conditions without blockade. Essentially the same relations were observed in 2 animals receiving hexamethonium and in one subjected to cardiac denervation. That is, guanethidine markedly increased both pressure and IST as judged by percentage change. In other experiments, atropinization did not affect the responses to guanethidine in acutely prepared vagotomized animals. Phentolamine totally blocked pressor response in one animal and limited the response to 17% above control in another; inotropic response in both these instances was the same as in dogs which had not received this adrenergic blocking agent.

Pressor and inotropic responses elicited by guanethidine in reserpinized animals averaged 30% and 21% respectively above control (Fig. 2). These responses are significantly less than with acutely prepared vagotomized dogs ($P < 0.001$). In contrast to the acutely prepared vagotomized animals, cardiovascular stimulation persisted only 4-10 min following injection. After subsidence of the cardiovascular stimulation, blood pressure and heart force were markedly below control.

Intra-aortic injections of guanethidine 5 mg/kg did not stimulate the adrenals as judged by circulating catecholamine levels. Similar injections of KCl raised epinephrine from 0.72 to 17.85 μg/l of plasma and norepinephrine from 1.41 to 9.74 μg/l. Neither arterial nor venous samples withdrawn during the period of stimulation after the drug's intravenous administration to vagotomized dogs showed a significant change from control.

Table I summarizes the content of catecholamines in ventricles and aortae of control and guanethidine-treated dogs.

A transitory decrease of 22% (S.D. ± 11.3%) in isotonic contractions of isolated rabbit hearts occurred when guanethidine was added to the perfusate. A mean increase

of 17% (S.D. ± 8%) persisted 5-10 min following the depression. A slight positive chronotropism paralleled the period of augmented contractile force. Repetitive doses rendered hearts less responsive to the drug. After guanethidine in 7 groups of 3 hearts, mean concentration of norepinephrine in pooled rabbit heart perfusate increased significantly from 0.17 to 0.94 μg/l of perfusate ($P<0.05$). Epinephrine increased insignificantly from 0.02 to 0.09 μg/l ($P>0.05$).

Discussion. Acute cardiovascular stimulation elicited by guanethidine in dogs with preganglionic blockade suggests the agent acts peripherally to autonomic ganglia and is not dependent upon sympathetic tone to elicit this stimulation. Significantly smaller responses in reserpinized animals, whose catecholamine and 5-hydroxytryptamine levels are diminished, indicates the mechanism may be dependent upon these cardiovascular stimulants. Inhibition of pressor responses to guanethidine by phentolamine indicates the mechanism is dependent upon catecholamines. Maxwell *et al.*(11) suggested that guanethidine depletes the adrenergic transmission agent from the post-ganglionic sympathetic fibers. In the present study, the immediate release of norepinephrine from isolated rabbit hearts as well as depletion of norepinephrine in ventricles and aortae of guanethidine-treated dogs supports this concept. Contemporary studies by Cass *et al.* (12) on depletion of norepinephrine from hearts of cats and rabbits treated with the agent also give a basis to this conception. Depletion of norepinephrine from peripheral sites suggests a reserpine-like action. To equal the degree of cardiovascular stimulation elicited by guanethidine, a large exogenous dose of a catecholamine is required. The fact that circulating catecholamine levels do not increase during the acute cardiovascular stimulation may be due to norepinephrine being released in proximity to end-receptors in quantities large enough to cause gross cardiovascular stimulation but not to discharge into the blood in measurable quantities.

MacIntosh and Paton(13) have shown that numerous organic bases similar to guanethidine release histamine. The initial hypotension seen after injecting guanethidine may be caused by histamine. The transitory nature of the vasodepression could be due to an antagonism of the depression by the pressor effect of the released norepinephrine. As the sources of norepinephrine become depleted after successive doses, the vasodepression is not as greatly antagonized; thus, it becomes more pronounced. Similar enhancement of vasodepression with successive doses of ephedrine have been reported(14). The possibility of a direct depressing action also exists and must be further studied.

Summary. Guanethidine elicits a period of augmented myocardial contractility. Significant decrements in ventricle and aorta norepinephrine content are caused by the drug. With isolated hearts, the agent releases norepinephrine.

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A New Approach to the Study of the Lymphocyte.* (26304)

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The lymphocyte is still an object of controversy. While some writers believe that it is an undifferentiated mesenchymal cell capable of developing into other cells(1,2,3) others feel that it is an end-form with a specific function and hence lacks prospective potencies(4,5,6). We have transferred large numbers of thoracic duct lymph cells from donor rats into granuloma pouches of recipient rats and studied these cells cytometrically and histologically. This new approach to the lymphocyte problem has been tested in over 100 recipient rats.

Method. Rats weighing 150 to 200 g were injected intraperitoneally or intravenously with 2.5 mg sodium pentobarbital/100 g of body weight, then anesthetized with sufficient ethyl ether to abolish the corneal reflex. Thoracic duct lymph cells were gathered through the left flank of the animals by a method similar to that described by Bollmann, Cain and Grindlay(7). After the peritoneal cavity was opened the dorsal peritoneum was incised transversely just below the diaphragm and the left kidney and adrenal were elevated and retracted medially. The need to strip the aorta and retract it separately was thus avoided and bleeding from its adventital vessels did not occur. The duct was opened just below the diaphragm by puncture with a 27 gauge hypodermic needle. Sharpened polyethylene tubing (#10 size) previously rinsed with 0.25% sodium heparin solution was inserted, tied in place with fine silk and held by ligature where it passed from the body wall. The wound was closed with clips and covered with gauze moistened in physiologic salt solution (PSS). The lymph was received in heparinized test tubes of 5 ml capacity fastened 10 to 15 cm below the level of the operating table. For the short time that lymph was collected the animal remained lightly anesthetized and tied to the operating platform. Hypodermoclycis with PSS was given during operation and collection.

Interscapular air pouches were prepared by the original method of Selye(8,9). For cytometric study, pouches of various ages were prepared by injecting 5 ml air into the interscapular region of 80 rats weighing 200 g. The age of the pouches ranged from less than 1 hour to 9 days. Pouches less than 1 day old received only 1 injection of air. One to 9 day old pouches were reinjected every 24 hours to maintain the same volume. Immediately after the last injection 1 ml of lymph, collected during the preceding hour, was introduced into the pouch. At various intervals from 15 minutes to 20 hours following injection of lymph, the rats were killed with ether. Five ml of saline were injected and the mixture was aspirated. Total and differential cell counts were done on the lymph before injection and on the saline-lymph mixture after withdrawal.

For histological study fresh pouches were similarly prepared by injection of approximately 3 cc of air in 34 rats weighing 150 g. Group A (7 rats) received air injection only. Group B (2 rats) received serum to which acridine orange had been added in a concentration of 1:5000 to test the affinity of tissue components for fluorochrome. In group C (7 rats), whole lymph was injected as soon as 0.5-1.0 cc had been collected. In group D (15 rats), cells to be identified in pouches by means of fluorescent microscopy were stained with acridine orange in concentrations from 1:10,000 to 1:100,000, washed with PSS using centrifugation at 1000 rpm, resuspended in PSS or dye-free lymph and injected into the pouch. Observations were made on motility, color of fluorescence and degenerative changes of the cells by means of blue light ultraviolet fluorescence using dark field illumination. Group E (3 rats) received cells stained with acridine orange and

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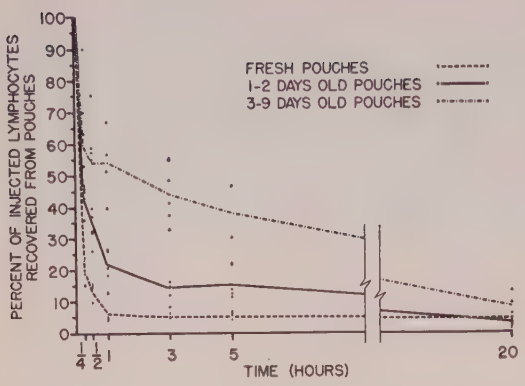

FIG. 1. Percent of inj. cells recovered by aspiration from pouches of various ages at various time intervals following inj. Lines shown on the graph are drawn through the means of groups of 3 to 6 rats.

killed by heat, ultraviolet irradiation or 1% ammonium molybdate solution. At intervals from ½ hour to 20 hours, the rats were killed with ether. Pouches dissected free of skin and most of the underlying muscle were fixed in Tellyesnicsky solution(10). Acridine orange stained pouches were placed first in freshly prepared 1% ammonium molybdate solution for 30 minutes following the principle of Baker(11). Tissues were imbedded in paraffin, semi-serial sectioned at 6 μ, deparaffinated, rapidly hydrated and mounted in glycerine. Luminous cells in the acridine orange groups were photographed by the light of a mercury vapor lamp filtered for blue light fluorescence, using a microscope equipped with Leitz UGI eyepiece filters. Photographic exposure was 5 to 10 minutes on 35 mm Kodak High Speed Ektachrome film (Daylight, ASA Index 160). The same section was then stained with hematoxylin and eosin for bright field examination and conventional photography.

Results. Placement of the tube in the thoracic duct was usually completed within 30 minutes after the rat was anesthesized. One ml or more of lymph containing 30 to 60 million cells was obtained in the first hour of collection. These figures are similar to those reported by Mann and Higgins(12). The lymph cells at time of injection showed no degenerative changes by dark field or phase contrast microscopy and when stained with acridine orange exhibited bright yellow fluorescence under the mercury vapor lamp.

The percentage of lymphocytes recovered by aspiration 15 minutes to 20 hours after injection into granuloma pouches of various ages is shown in Fig. 1. Sections of the walls of fresh and 7-day-old pouches are shown in Fig. 2A and B. In fresh pouches only 20% injected cells were recovered in 15 minutes, 5% in 3 hours and 4% in 20 hours. In 3-9 day old pouches cells disappeared much less rapidly. In 1-2 day old pouches the figures were intermediate.

While the lymphocytes disappeared from the lumen, polymorphonuclear leukocytes migrated into it. In pouches of all ages total number of polymorphonuclear leucocytes found in the saline-lymph mixture rose from an average of 1.06 million after 15 minutes to 3.25 million after 3 hours and to 5.32 million after 20 hours. Monocytes on the other hand did not change significantly. It is true that after 5 hours the saline-lymph mixture contained an average of 10% and after 20 hours 17% monocytes as compared to 6% after 15 minutes. The total numbers, however, were 1.23 million after 15 minutes, 1.08 million after 5 hours and 1.12 million after 20 hours.

Only freshly prepared pouches similar to Fig. 2A were used for histologic studies. No leukocytic infiltration was seen in pouches injected with air only (Group A). Following injection of serum, whole lymph or lymph cell suspensions both polymorphonuclear leukocytes and monocytes appeared in the wall. Very few lymphoid cells were found within 20 hours after serum injection. Injected lymph cells (Groups C and D) occupied the lumen and inner portion of the pouch wall (Fig. 2C). They were readily distinguished from the host's polymorphonuclear and monocytic reaction cells. Lymph cells stained with acridine orange (Group D) appeared brilliant yellow in fluorescence microscopy of the sections in contrast to the faint autofluorescence of the tissue (Fig. 2D). Injected cells which had adhered to and entered the wall of the pouch were distributed irregularly. They were seen chiefly in the mesenchyme between the lobules of

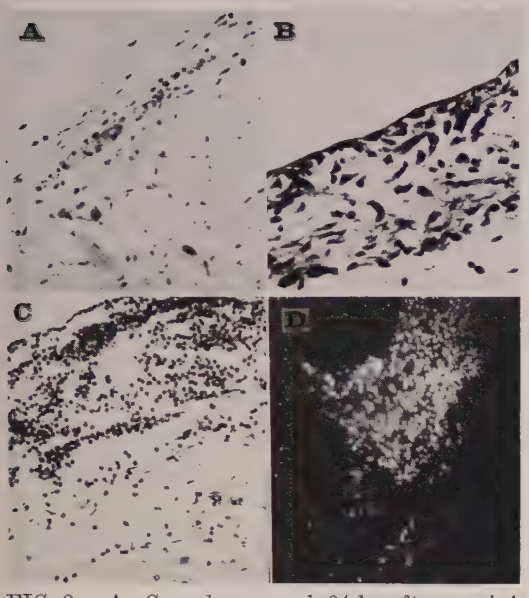

FIG. 2. A. Granuloma pouch 24 hr after one inj. of air, hematoxylin and eosin (H & E). B. Granuloma pouch after 7 daily inj. of air (H & E). C. Lymph cells 4 hr after inj. into air pouch prepared 10 min. previously (H & E). D. Fluorescent acridine orange stained lymph cells 1 hr after inj. into air pouch. Photomicrograph by blue light fluorescence with UGI eyepiece filter and 8 min. exposure on High Speed Ektachrome (copy from 35 mm slide). Lumen of pouch seen in upper left corner.

fatty tisue. Depth of penetration and structure of acridine orange stained cells was indistinguishable from that of the unstained Group C cells but because of the fluorescence in Group D these were more easily found deep in the pouch wall. At ½ hour, only a few of the injected cells appeared in section to have degenerative changes. After 6 hours, considerable nuclear fragmentation and lysis were seen in those cells which remained adherent to the inner surfaces of pouches. Group E cells killed with heat or ultraviolet light liberated dye which transferred to other tissue elements. Fluorescent serum (Group B) produced similar tissue fluorescence. Nuclei of ammonium molybdate killed cells, on the other hand, were identified within the pouch 18 hours after injection, were not seen in the pouch wall and did not impart appreciable fluorescence to other structures. Orange fluorescing granules were found in germinal centers of the axillary nodes of some Group D animals. Whether this represented nuclear material from degenerated injected cells or dye transfer is not known.

Discussion. Of the possible sites in which the lymphocyte may change into another cell or perform a specific function, tissue seems more likely than either blood or lymph. The wide dispersion of tissue lymphoid cells and the problem of positive identification has prevented effective observation or counting. The method which we have described provides a high concentration of thoracic duct lymph cells in the lumen and wall of a reproducible, dissectable connective tissue pouch. It is generally accepted that normal thoracic duct lymph contains over 90% small lymphocytes while the remainder are almost entirely intermediate and large lymphoid cells which may be safely counted as lymphocytes(2). The simple air pouch used here is an air bubble within loose interscapular connective tissue. The air in the pouch is moist, near body temperature and in gas equilibrium with the tissue of the host. At least in the fresh pouch, there is no cellular barrier between injected lymph cells and connective tissue.

Collection of thoracic duct lymph causes little or no cell damage. The preserved structure, motility, and color of cell fluorescence just before injection indicate that the procedures used have not killed many of the cells. Rate of disappearance from the lumen and depth to which cells have penetrated the pouch wall within the first 4 hours also suggest actively motile cells, particularly so because molybdate killed, thoroughly washed cells remained in the lumen after 18 hours. In one animal the polyethylene tubing was led from the thoracic duct directly into an interscapular pouch through a needle. Tissue sections obtained from this preparation were very similar to those from pouches injected with washed acridine orange stained lymph cells. Many abnormalities were seen when cells aspirated from pouches were smeared and stained. Although this may be partly the effect of the recovery procedure and smear technic, it also seems likely to us that the cells which failed to enter the pouch wall were the less actively motile cells. These may represent the 5% which could be recovered after 1 hour from fresh pouches or at

20 hours from pouches of various ages.

About 40% of the residual fluorescent cells aspirated ½ hour after injection into pouches similar to but not included in Group D showed fading of stain, cytoplasmic and nuclear alteration and orange fluorescing granules resembling Gall bodies. In Wright-stain smears, these specimens resembled non-fluorescent aspirates. Because of these considerations and the apparently identical behavior of unstained and acridine orange stained cells in tissue, we have not carried out a complete series of aspiration studies with vitally stained cells. Chemical fixation (trapping) of acridine orange within the vitally stained cells is essential to subsequent histologic study. As the fluorochrome gradually leaves the living cell and ammonium molybdate is toxic, other methods of labeling may prove preferable for either histologic or cytologic studies of long duration.

The transfer of thoracic duct lymph cells into granuloma pouches as described permits simultaneous injection of antigen, fatty substances and other materials which are known to stimulate development of plasma cells, macrophages, and other cells. Also either donor or pouch animal can be pre-treated to alter the lymph cells or their tissue environment.

The fate of transferred lymphocytes has not yet been determined. Some of the cells disintegrated. The difference in disappearance rates between fresh and old pouches (Fig. 1) however, indicates that disintegration was no major event. Many injected lymphocytes migrated through the connective tissue surrounding the pouches. This was seen especially in fresh pouches surrounded by loose connective tissue. There was no evidence of transformation of lymphocytes into monocytes in these short-term experiments or those previously reported by us (13).

Summary. A new approach to the study of the small lymphocyte is presented. Thoracic duct lymph cells from donor rats are injected into granuloma pouches in recipient rats and the transferred cells are studied both cytologically and histologically. Advantages and limitations of the new method are mentioned.

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Effect of Thermal Injury on Ascorbic Acid and Tyrosine Metabolism. (26305)

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It is well established that stress alters many metabolic patterns of the animal organism extensively(1). The metabolism of Vit. C has been studied in this context, and it appears that a severe injury imposes a need for Vit. C far above normal. This conclusion is based on studies of Vit. C tissue saturation and blood levels, and wound healing(2). The present investigation was aimed at determining whether the increased need for Vit. C after stress could be demonstrated for an enzymatic function in which this vitamin is known to be involved, namely, metabolism of tyrosine.

Garrod(3) made a significant contribution to the theories of the metabolism of the aromatic amino acids by correctly ascribing alcaptonuria to an inborn error of metabolism. Alcaptonuria is marked by appearance in the urine of homogentisic acid (2,5 dihydroxyphenylacetic acid) and is caused by a lack or absence of the enzyme necessary to carry the conversion of tyrosine further. It was subsequently discovered almost simultaneously by Sealock(4) and Levine(5) that Vit. C is required for formation of homogentisic acid from its precursors. Sealock(6) also showed conclusively by differential colorimetry and by preparation of derivatives, that the urinary acids resulting from feeding l-tyrosine or l-phenylalanine to scorbutic guinea pigs are primarily p-hydroxy phenylpyruvic acid and smaller amounts of homogentisic acid; this established that the metabolic defect of scurvy was fundamentally different from inherited alcaptonuria.

The present study was designed to answer the following: (A) Does a severe burn cause appearance of abnormal urinary compounds even when the animals are provided 2 mg/day Vit. C, an amount known to be sufficient for normal growth and wound healing(2,7); (B) if so, what is the nature of the compound or compounds; and (C) can this be prevented by administration of larger doses of ascorbic acid?

Materials and methods. Male guinea pigs (200 ± 10 g) were used, obtained from Fort Detrick, Md. (Hartley strain). They were offered, *ad libitum,* a ground Rockland* diet that was nutritionally complete except for a total lack of Vit. C. The diet was supplemented (except for those animals which were meant to become Vit. C deficient) with 2 mg ascorbic acid in 0.5 ml water administered each day by pharyngeal tube.

The guinea pigs were housed in metabolic cages in an air conditioned room, the temperature of which was kept at 78° ± 1°F. The cages were provided with plastic funnels, with glass urine-feces separators attached. Each day, cages and accessories were cleaned and the water changed.

The tyrosine load test was performed as follows: 0.75 mg l-tyrosine per gram of body weight was administered by stomach tube as a slurry in approximately 10 ml of 0.9% saline.

Urine was collected on a 24-hour basis. Each collector contained 5 ml of 2 N hydrochloric acid, an amount sufficient to maintain the urine acid to litmus at all times. Each collection was filtered to remove solid particles, the filter paper washed twice with water, and the filtrate was diluted to 100 ml. An aliquot of one-tenth of this volume was extracted 3 times into ethyl ether. This step excluded all inorganic compounds and organic basic compounds. The ether phase was reextracted into 5% sodium bicarbonate solution. This step excluded weak acids, such as phenols. Finally the sodium bicarbonate solution was acidified with hydrochloric acid and extracted into ether. The ether was

* Obtained from the A. E. Staley Mfg. Co., Decatur, Ill.

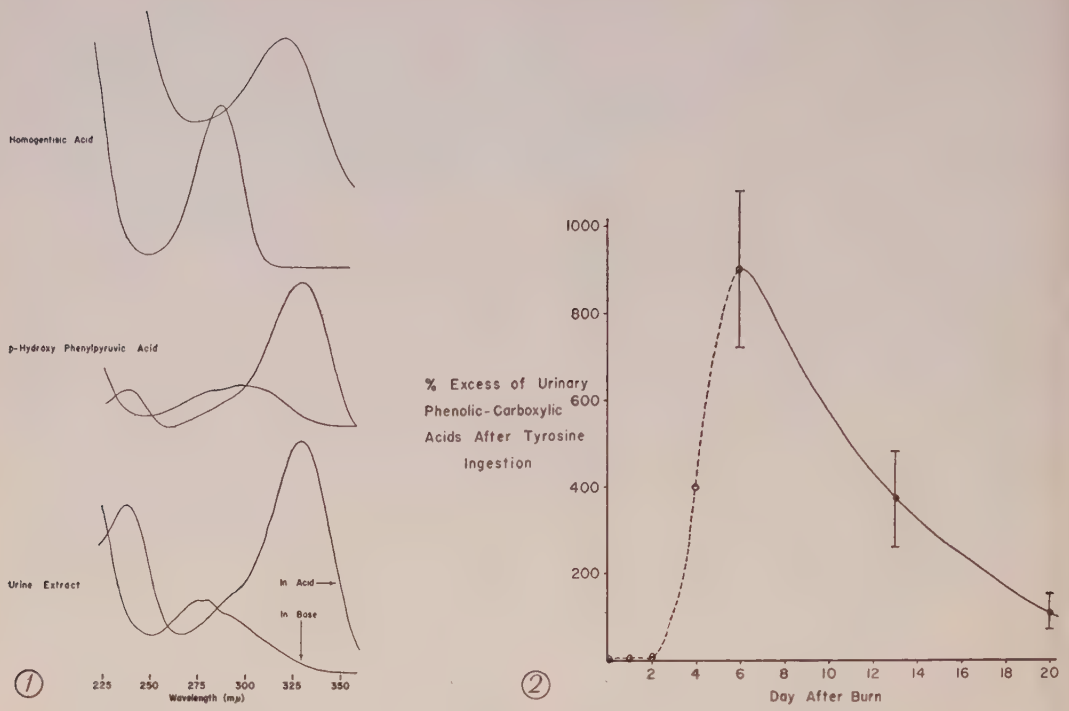

FIG. 1. Ultraviolet spectra of urine extract of burned guinea pig, p-hydroxy phenylpyruvic acid, and homogentisic acid, all in acidic and basic water solutions.

FIG. 2. Effect of burn on tyrosine metabolism: Percent excess of phenolic-carboxylic acids in 24 hr urine after tyrosine ingestion (0.75 mg/kg body wt) compared with 24 hr urine before tyrosine ingestion. All guinea pigs were maintained on 2 mg/day vit C. Dotted line denotes single guinea pigs burned at 73°C; solid line, 19 animals burned at 85°C. Vertical lines on the curve denote stand. error.

evaporated to dryness and the residue taken up in 25 ml absolute ethanol. This procedure could be expected to isolate strongly acidic organic substances, including carboxylic acids and carboxylic phenols. The alcoholic solutions were then examined spectrophotometrically at 297 mμ, using a Beckman DU instrument. Spectra of pure compounds and, in some cases, urine extracts were obtained with a Cary Model 11 recording spectrophotometer.

Some animals were subjected to a standard back burn under ether anesthesia(8). Burns were produced by dipping the guinea pigs' backs into water containing a wetting agent, polyoxyethylene lauryl alcohol.† The burns were administered at 73° or 85°C, involved one-third of the body surfaces and were third degree. Half the animals were treated immediately post burn with 0.9% saline and half with 0.9% saline containing 5% dextrose. These solutions were administered by stomach tube in an amount equal to 5% of the body weight. There were no significant differences in survival rates of these groups (one of each group died), or their tyrosine tests. The tyrosine test results, therefore, have been combined for presentation.

Results and discussion. Fig. 1 shows the ultra-violet spectra of urine obtained from a burned guinea pig after tyrosine feeding, and of 2 pure compounds known to be formed *in vivo* from tyrosine *i.e.*, homogentisic acid and p-hydroxy phenylpyruvic acid. All 3 show the transposition and splitting of maxima characteristic of phenols. The urine extract spectrum seems to be a composite of the 2 compounds, with p-hydroxy phenylpyruvic acid greatly predominant. Small amounts of other acidic aromatic compounds may, of

† BRIJ 35, obtained from Atlas Powder Co., Wilmington, Del.

course, also be present. At any rate, homogentisic acid is at most a minor component. These results show that tyrosine metabolism is altered as a result of a severe burn, and that the abnormality is probably due to a derangement of Vit. C metabolism, since the abnormal urinary components of the burned guinea pig are apparently identical to those found in the urine of scorbutic guinea pigs.

Sealock and Silberstein(6) showed that fed tyrosine yielded urinary phenolic acids if Vit. C was omitted from the diet of the guinea pig for only one day. Painter and Zilva(9) confirmed this, and we also observed the same rapid onset of the abnormality in experiments preliminary to the present study.

Two groups of workers(10,11) have now demonstrated that livers of Vit. C deficient guinea pigs which had been given excess tyrosine have both an increase in activity of the enzyme system producing p-hydroxy phenylpyruvic acid and a decrease in activity of the enzyme system which oxidizes it, thus accounting for its accumulation in those animals.

In addition, McElroy and Anderson(12, 13) have shown that tourniquet injury inflicted on rats results in a 3-fold increase in ability of the isolated rat liver to produce p-hydroxy phenylpyruvic acid from tyrosine. They concluded that this striking increase in activity was due to suppression of an inhibitor to tyrosine α ketoglutarate transaminase, rather than to an increase in the transaminase itself, and that rate of reaction of tyrosine with pyridoxal phosphate (the first step in transamination), was thereby specifically increased.

These findings are consistent with those of Knox(10) and Zannoni(11) and implicate derangements of both p-hydroxy phenylpyruvic acid producing and degrading systems, whether due to ascorbic acid deficiency or to some other cause.

Fig. 2 shows results of our experiment involving 19 burned guinea pigs. The figures on the abcissa refer to the day just before administration of test dose, and the day just after. All urines were also analyzed the second and third days after the tyrosine test, but were invariably normal.

In view of the heterogeneity of the compounds we measured we have not attempted to relate our absorbency units to a specific compound, but have calculated total "optical density" units for each day's urine from the aliquot which we took for analysis. The data show conclusively that the burn creates an additional need for Vit. C beyond the 2 mg/day which are normally sufficient to maintain the enzymatic systems necessary for tyrosine metabolism. This requirement had been established by preliminary experiments. The metabolic derangement reaches its peak on or near the sixth day after the burn, and declines almost linearly thereafter. One guinea pig, not represented on this graph, was maintained on 100 mg/day Vit. C after the burn, and did not react abnormally to the tyrosine test performed on the fifth post-burn day. Further work is required to establish minimum Vit. C supplement necessary to abolish the abnormal post-burn response.

This pattern of metabolic reaction to injury as it involves Vit. C agrees with that previously observed by Levenson, *et al.*(2), who found that healing of wounds of burned guinea pigs was strikingly abnormal during the first postoperative week. These wounds were histologically indistinguishable from those of guinea pigs depleted of Vit. C by nutritional deprivation. The abnormalities were still present 10 days postoperatively, though to a lesser extent, and by the fourteenth day, the wounds were almost normal. These animals received 2 mg Vit. C per day. Addition of 100 mg/day Vit. C to the basal diet allowed the wounds to heal normally, despite the superimposed burn. The authors ascribed the abnormal wound healing to derangement of ascorbic acid metabolism induced by the burn. Our findings confirm this hypothesis.

Summary. Intragastric administration of 0.75 mg of l-tyrosine per g body weight to guinea pigs maintained on 2 mg of Vit. C per day did not yield urinary tyrosine intermediate metabolites. Burned guinea pigs to which this test was applied at various times after the burn showed an abrupt abnormal response on the third post burn day despite

a daily maintenance Vit. C dose of 2 mg per day. This response was manifested by appearance of large amounts of urinary p-hydroxy phenylpyruvic acid, the same compound which appears in the urine of uninjured guinea pigs fed tyrosine when ascorbic acid is removed from their diet. Metabolic abnormality reached its peak on the sixth post burn day, then declined to almost normal levels by the 20th post burn day. One hundred mg per day of Vit. C abolished the abnormality after the burn. These results agree with previous observations of altered Vit. C metabolism and an increased need for this vitamin after burns, as judged by the abnormal wound healing within the first 2 weeks after a burn.

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A Method for Measurement of Urinary Deoxyribonuclease Based on the Reaction of Indole with Deoxyribonucleotides.* (26306)

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The need for a more precise sensitive method to determine the activity of urinary deoxyribonucleases I and II (DNase I and DNase II) has led us to develop such a method based upon the indole reaction originally described by Dische(1) and used by Ceriotti for estimation of deoxyribonucleic acid(2). The failure to obtain good reproducibility even with zero time blanks, as noted with other urinary DNase methods(3,4), has been eliminated by insuring the quantitative precipitation of unreacted DNA. This has been accomplished by adding a protein such as casein or bovine serum albumin to the enzyme reaction mixture.

The use of purified, freshly distilled chloroform in Ceriotti's procedure has been circumvented by substituting commercially available analytical grade benzene as extracting solvent.

Insight into the mechanism of the indole reaction has been gained by comparing reactivity of purine and pyrimidine deoxyribotides and the corresponding deoxyribosides as well as that of certain possible hydrolysis products of DNA.

The method developed primarily for measurement of urinary DNase activity has been found equally applicable to measurement of DNase in serum and tissue homogenates. However, only a general method for determination of DNase I will be discussed here.

Experimental. I. *Reagents.* A. *General reagents for DNase I and II*: (a) A 0.2 g% solution of sodium deoxyribonucleate (DNA). DNA was prepared in this lab-

* Supported by U. S. Public Health Service grant.

oratory by the method of Kay, Simmons, and Dounce(5). (b) 2.88 M trichloroacetic acid (TCA). (c) 0.04 g% indole in distilled water(2). (d) Concentrated (12 N) HCl. (e) Benzene, thiophene free (C. P. Mallinckrodt has been found satisfactory without further purification).

B. *Reagents for DNase I*: (a) A buffer solution, pH 7.5, made by mixing 75 ml of 0.2 M tris (hydroxymethyl) aminomethane with 100 ml 0.1 M HCl, adjusting the pH to 7.5, and diluting to 300 ml with distilled water; this solution is then mixed with 100 ml 0.1 M magnesium chloride. (b) A 1.0 g% solution of Vitamin test casein (General Biochemicals) was prepared by mixing 1 g casein with 20 ml of water and adding 1 N NaOH (usually 11 to 12 ml) until the casein dissolved completely. This solution was then neutralized (pH 7.0) by adding 11 to 12 ml of 1 N acetic acid dropwise with continuous stirring. The casein solution was then diluted to 100 ml with the above pH 7.5 buffer.

II. *Methods for collection and storage of urine*: Urine was collected as a clean, single, freshly voided morning specimen (average interval, 8 hours). Under these conditions, the use of toluene as an antiseptic did not yield significantly different results and its use was eliminated. A 25 ml portion of the total measured volume was placed in a 30 cm segment of dialysis tubing (cellophane, 28 mm diameter, 0.18 mm wall thickness) weighted with a glass marble. Dialysis was continued for 24 hours. If the urine was not dialyzed before analysis, so much extraneous pink pigment was produced in the reaction with indole that 12 to 15 extractions with benzene were necessary. All urines were dialyzed immediately after collection. For dialysis, running cold tap water was used because no significant difference between tap water and distilled water dialysis was observed. After dialysis, the urine was immediately frozen and stored until assayed. Routinely, analyses (for both DNase I and II) of a given thawed specimen were run the same day. The stability of DNase I activity after freezing and thawing was tested on 8 urines; there was no significant change in activity after one thawing; after subsequent refreezing and thawing, as much as 50% of the activity was lost.

III. *General DNase assay method*: A mixture of 1.0 ml of the appropriate buffer, 1.0 ml of the specimen,† 1.0 ml of DNA solution, and 1.0 ml of a specific protein solution such as casein was incubated at 37.5°C for varying lengths of time as noted below. One ml of 2.88 M TCA was added to terminate the incubation and the resulting suspensions were filtered through Whatman No. 40 filter paper (7 cm diameter). Appropriate controls were set up in which reagents were mixed at zero time or in which the reaction was terminated at 1½ hours (urinary DNase I and serum DNase I respectively). All determinations except the zero time controls were carried out in duplicate. Two ml aliquots of the filtrate were then mixed with 1.0 ml of indole solution and 1.0 ml of HCl, heated in a boiling water bath for 10 minutes, cooled to room temperature with the aid of a cold water bath, and extracted repeatedly (usually 2 or 3 times) with 6 to 8 ml aliquots of benzene (used in place of the chloroform of the Ceriotti method(2)) until the benzene layer was colorless. Extraction with benzene was necessary to remove any pink-colored material which otherwise interferes with the final colorimetric measurement. The benzene was removed each time with the aid of suction. After extraction, the somewhat hazy yellow aqueous layers were clarified by centrifuging (International type SB, No. 1) at 2000 rpm for 10 minutes, and read at 490 mμ with the Beckman spectrophotometer against a blank solution containing 2 volumes of the appropriate buffer, one volume of TCA, and one-half volume of water. Against this blank solution the reaction zero time blanks with urine had optical density values generally less than 0.050.

By comparison with the optical density value obtained for a 10 μg and a 20 μg deoxy-

† In the assay method for human urinary DNase I, 1.0 ml of dialyzed diluted urine was incubated for one hour with 1.0 ml of 1% casein. Since undiluted urine was too active as such, human urine was diluted 1:20 with distilled water.

TABLE I. Reactivity of Deoxynucleotides and Related Substances with the Indole Method.*

Substances reactive with indole			Substances slightly reactive with indole			Substances non-reactive with indole	
Substance		O.D.	Substance		O.D.	Substance	O.D.
Deoxyadenylic acid, diammonium salt	(8.05)	.882	Deoxyadenosine	(.15)	.024	Cytidylic acid	.004
			Deoxyguanosine	(.13)	.020	Cytosine	.004
Deoxyguanylic acid, calcium salt dihydrate	(7.85)	.740	Thymidine	(.09)	.015	Deoxycytidine · HCl	.006
			Deoxyuridine	(.07)	.012	Guanine	.003
			2-Deoxy-D-ribose	(.12)	.036	Glucoseamine · HCl	.002
Thymidylic acid, calcium salt dihydrate	(1.91)	.193	Fructose-6-phosphate, barium salt	(.14)	.014	Adenosine	0
						D-(−)-ribose	.003
						Adenine · SO_4	.004
Deoxycytidylic acid	(0.49)	.064	Hexose diphosphate, calcium salt	(.11)	.010	Glucose-1-phosphate, dipotassium salt	.001
			Ribose-5-Phosphate, barium salt	(.09)	.010	Glucose-6-phosphate, barium salt	.002
						Barium phosphoglyceric acid	0

* Optical densities of 40 μg standards are given. (Values in parentheses are computed micromolar extinctions.)

adenylic acid (dAMP) standard‡ treated identically and run with each set of unknown specimens, the activity of the specimen in question was calculated and the results expressed in units defined as follows: One *unit* of *DNase* activity is equivalent to that amount of TCA-soluble nucleotides liberated during the selected incubation time and reacting with indole to the same extent as 1.0 μg of dAMP. In the case of urine, activity may be expressed in units per hour of urine collection.

IV. *Reactivity of deoxynucleotides and related substances in the indole reaction*: A stock solution of 500 μg per ml of 10% TCA was made of the following (all obtained from California Corp. for Biochemical Research): (1) Deoxyadenylic acid, diammonium salt (dAMP). (2) Deoxyguanylic acid, calcium salt dihydrate (dGMP). (3) Thymidylic acid, calcium salt dihydrate (dTMP). (4) Deoxycytidylic acid (dCMP). Solutions of 5, 10, 20, and 40 μg of each of the above per ml of 10% TCA were made from the stock solutions. Two ml aliquots of these dilutions were then treated with indole according to the procedure described above.

In addition, a variety of substances (Table I) related to the nucleotides and nucleosides as possible hydrolytic products or as substances of similar chemical structure were tested for their reactivity with indole. Standard solutions of 10, 20, and 40 μg per ml of 10% TCA were made and treated with indole as described above.

Studies were carried out with dAMP to determine the effect of time of heating in the boiling water bath on optical density at 490 mμ and to ascertain to what extent the color reaction was dependent upon time of addition of indole. These experiments were as follows: (a) Duplicate 20 μg dAMP and dGMP standards were treated with indole and HCl as above and heated in the boiling water bath for different time intervals from 2 to 40 minutes. Duplicate 20 μg dTMP and dCMP standards were treated in the same way, except that they were heated in the boiling water bath for 10, 20, 30, and 40 minutes. (b) Duplicate 10 and 20 μg dAMP standards were heated with HCl alone for the same time intervals as above. At the end of these times, indole was added and they were heated for 10 minutes longer. (c) Duplicate 10 μg dAMP standards were incubated at 37.5°C with HCl for time intervals of 2 to 30 minutes. At the end of these times indole was added and the mixtures were then heated for 10 minutes in the boiling water bath.

‡ For this purpose a stock solution of 500 μg of dAMP per ml of 10% TCA was made and stored in the refrigerator where it remains stable for months. This solution was diluted with a solution of essentially the same composition as the reaction mixture minus DNA to give a final concentration of 10 μg and 20μg of dAMP per 4 ml of the final indole reaction mixture.

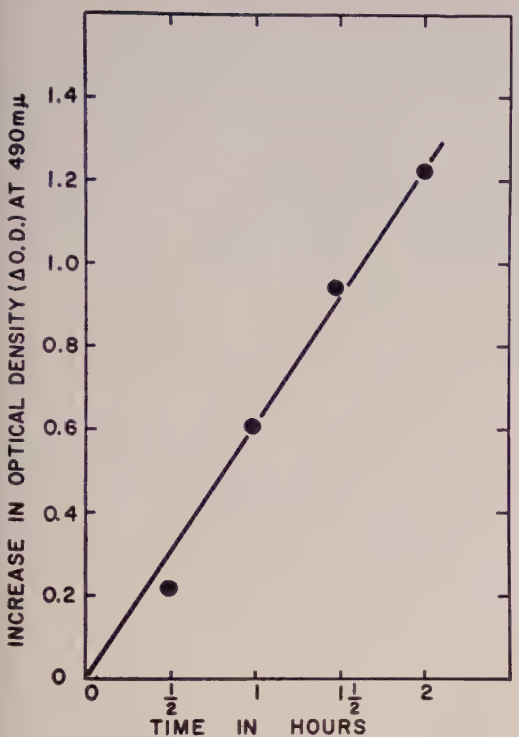


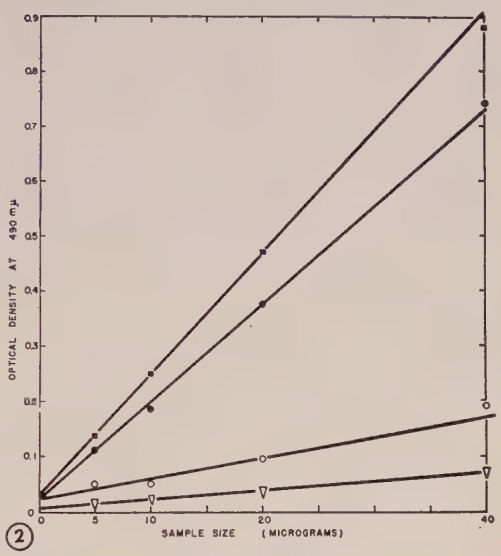
FIG. 1. DNase I activity (pH 7.5)—Human urine (1:20).

To compare the indole reaction with other methods for measuring urinary DNase activity, aliquots of the same TCA filtrates were examined by the diphenylamine method of Dische(6) and by the method of Schneider and Hogeboom(4).

Results and discussion. The reaction of indole with deoxyribonucleic acid first described by Dische(1) and used by Ceriotti (2) as the basis of a method for measuring DNA has been studied and affords a sensitive basis for quantitative measurement of the enzyme's urinary DNase I and DNase II. Fig. 1 shows the typical linear increase in TCA soluble hydrolytic products resulting from the action of human urinary DNase I on DNA as estimated by the indole reaction.

Early in the development of the indole reaction to measure urinary DNase activity, it was noted that the TCA filtrates were faintly hazy even in the case of the zero time controls, and that they gave poor duplicates and generally high values in the indole reaction. To precipitate what appeared to be colloidal DNA or apurinic acid, leaving only relatively low molecular weight fragments soluble in TCA, bovine serum albumin was added to the zero time controls and to incubation mixtures immediately before stopping the reaction with TCA. This gave crystal clear filtrates and consistently low zero time controls. After several other experiments with more readily available proteins, casein was chosen for use with the DNase I assay.

Fig. 2 reveals that in 10 minutes the purine



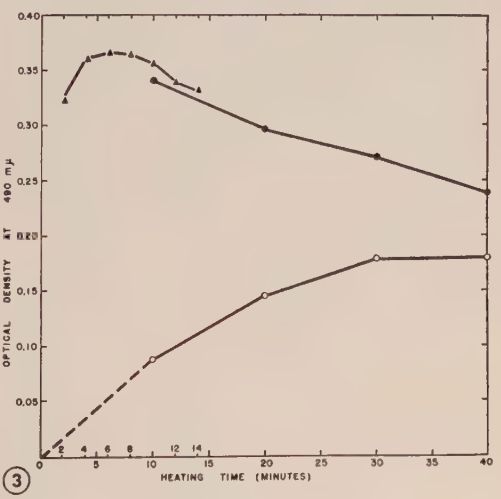


FIG. 2. ■—■ dAMP; ●—● dGMP; ○—○ dTMP; △—△ dCMP.

FIG. 3. ▲—▲ dAMP (20 μg/ml); ●—● separate determination of dAMP (20 μg/ml); ○—○ dTMP (20 μg/ml).

nucleotides react with indole to an extent more than 4 times as great as the pyrimidine nucleotides. When the absorption spectrum of the yellow solutions from the indole reaction with each of the nucleotides was plotted, identical curves were obtained with a prominent maximum at 490 to 495 mμ. These curves were similar to that described by Ceriotti(2) for DNA. Fig. 3 illustrates that maximum reaction of the dAMP standards heated with indole and HCl was reached in 4 to 10 minutes, and that further heating resulted in loss of activity. Fig. 3 also shows that with dTMP the extent of the indole reaction increases up to 30 minutes, then levels off. Although not shown in Fig. 3, dGMP and dCMP behaved qualitatively and quantitatively like dAMP and dTMP respectively.

Thus, where purine nucleotides have reached a maximum reaction within 10 minutes time, pyrimidine nucleotides do not yield a maximally intense color before 30 to 40 minutes. Since the color produced from purine nucleotides is destroyed to an appreciable extent by heating beyond 10 minutes time, the possible increase in sensitivity that one might anticipate from more protracted heating of the pyrimidine nucleotides is counterbalanced. This, in effect, explains why the 10 minute heating period selected by Ceriotti is optimal for DNA.

Heating dAMP with HCl alone from 2 to 40 minutes at 100°C completely destroys its reactivity with indole since further heating for 10 minutes after addition of indole yielded no characteristic color absorbing at 490 mμ. However, incubation of dAMP at 37.5°C with concentrated HCl alone, and then, after addition of indole, heating in the boiling water bath in the usual manner resulted in as much color development as was obtained in the samples treated in the routine manner.

The reactivity of the deoxynucleotides is in sharp contrast to the insignificant activity of the corresponding deoxynucleosides and to the minimal activity of 2-deoxyribose itself. Quantitatively, on a molar basis, the reactivity of 2-deoxyribose is less than 2% that of the purine nucleotides. The virtual complete lack of reactivity of the purine or pyrimidine bases and of the purine and pyrimidine nucleosides strongly suggests that the reactive substance derived from deoxyribonucleic acid is deoxyribose-5-phosphate or some precursor readily giving rise to deoxyribose-5-phosphate in the presence of HCl. The clear demonstration of the reactivity of an authentic specimen of deoxyribose-5-phosphate with indole in presence of HCl is necessary before this presumptive conclusion is proved.

In comparing the results obtained from the indole method with those obtained from the other methods tested, it was found that a given amount of DNA hydrolytic products gave a substantially greater optical density in the indole reaction than was observed with either of the other two methods. In an experiment using a rat spleen homogenate as a DNase source, optical density values obtained on the same aliquot of TCA filtrate from a given DNase reaction, with the indole reaction, Schneider and Hogeboom's technic and Dische's diphenylamine procedure, were 0.529, 0.386, and 0.130 respectively.

The reaction of indole with the deoxyribonucleotides and the failure of the corresponding nucleosides or free deoxyribose to react afford a basis for a simple sensitive quantitative test for measurement of deoxyribonucleotides in presence of the corresponding phosphate free compounds.

Summary. A method is described for measuring urinary deoxyribonuclease activity. The method is based on the reaction of indole in presence of HCl with the products of DNA cleavage. The reaction has been found specific for deoxyribonucleotides and occurs to an insignificant extent with deoxyribonucleosides, free purines or pyrimidines, deoxyribose, and a variety of sugar phosphates other than deoxyribose-5-phosphate.

The authors wish to thank Mrs. Leon L. (Elizabeth P.) Miller, Miss Gilda Guarasci, Mrs. Elizabeth Arrowsmith for their technical assistance, Dr. Thomas Koszalka for his advice, and Mr. Donald Gordon for providing us with normal serum.

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Cardiac Temperature Gradients During Profound Hypothermia with Extracorporeal Perfusion.* (26307)

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Whether or not cardiac temperature gradients during induction of hypothermia and rewarming are related to onset of ventricular fibrillation remains equivocal. That such differences in temperature do occur has been reported by Brooks(1), but no experimental data have been presented. He further added that " . . . such studies have not been carried far enough to justify statements other than that marked gradients are present when the heart is susceptible to fibrillation or is difficult to defibrillate." Prior to this Hegnauer (2) speculated that such gradients would be expected and might be responsible for ectopic activity in the ventricles. Because of the paucity of information relevant to cardiac temperature variations and fibrillation when either immersion or direct blood cooling was used to produce hypothermia, such a study was undertaken using the latter method in conjunction with extracorporeal perfusion.

Materials and methods. Fourteen mongrel dogs of both sexes, weighing 12 to 20 kg, were anesthetized with pentobarbital sodium, 25 mg/kg, attached to a Medtronic automatic respirator and connected to an extracorporeal system (consisting of a T. M.-2 Sigmamotor perfusion pump, a 13″ convoluted disc Kay-Cross oxygenator, and a gas type heat exchanger)(3) which permitted control of cooling and rewarming. The unit was primed with heparinized whole blood. An inflow catheter was inserted *via* a femoral artery into the aorta at the level of the renal arteries, and outflow catheters were placed in the vena cavae through femoral and jugular veins. Prior to cooling a thoracotomy was performed and 5 or 7 copper-constantan thermocouples† were implanted in various sites of the heart. Following closure of the thorax a similar thermocouple was passed orally into the mid-esophagus. The precise position of all thermocouples was confirmed by sacrifice of animals at conclusion of each experiment. Temperatures were automatically registered every 30 seconds on a Leeds-Northrup Speedomax Type G recorder. Variations of temperature as slight as 0.1°C could be detected. Cardiac activity was continuously monitored by a Sanborn Visoscope and recorded intermittently on a Sanborn Polyviso. The perfusion rate during cooling and rewarming was maintained at 50 cc/kg/min. Cooling was continued until the esophageal temperature registered 10°C, at which time rewarming was instituted. Perfusion was discontinued when esophageal temperature was between 25 and 30°C.

Results. Cardiac temperature differentials were noted in every animal studied. Four examples, each from a separate animal, representing the usual variations observed are shown in Fig. 1. Although temperatures were simultaneously obtained from sites other than those illustrated, for clarity of presentation they were omitted when they were similar or close to the ones that were used. While temperatures recorded from vari-

* Supported by U.S.P.H.S. grant, Dept. of the Army Contract and Southwestern Pennsylvania Heart Assn.

† Type NN-30-DT-Cu-Constantan-30 gauge BNS, Nylon Insulation—Thermo Electric Co., Inc., Saddle Brook, N. J.

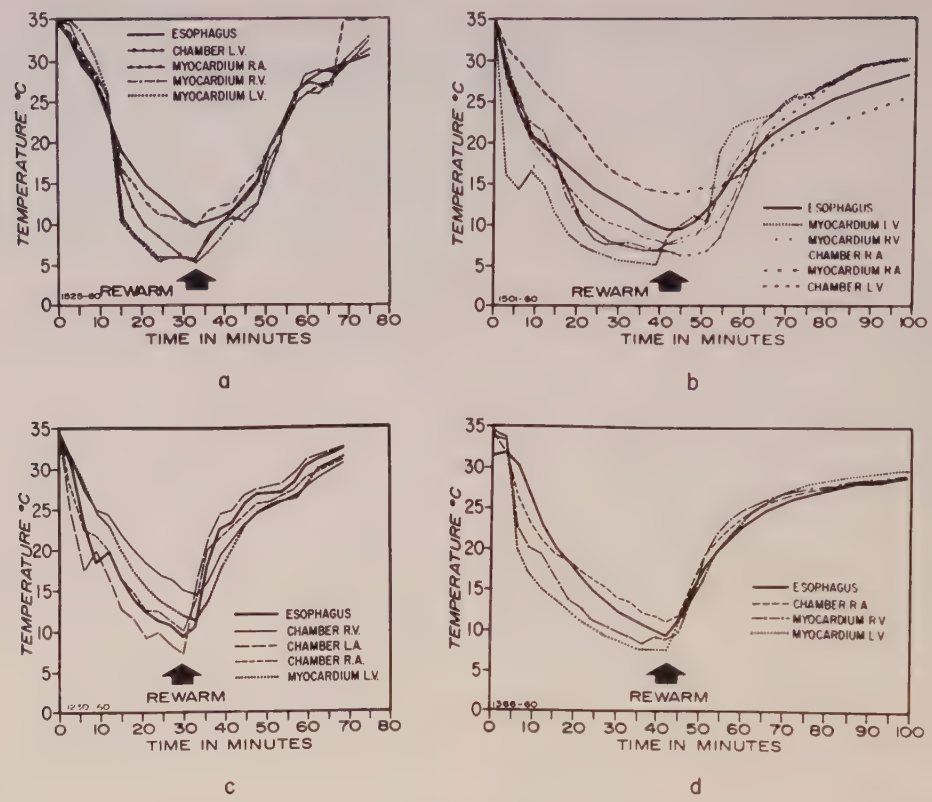


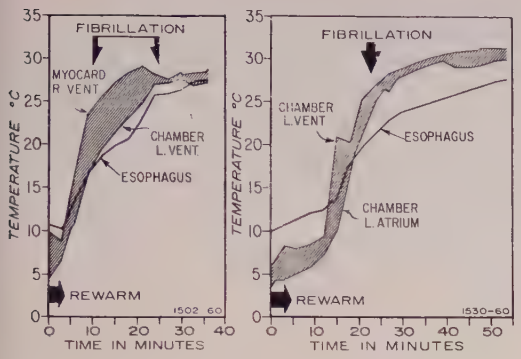
FIG. 1. Cardiac temperature gradients in 4 representative animals subjected to profound hypothermia and rewarming with extracorporeal perfusion.

ous parts of the normothermic heart showed little variation, with onset of cooling differences were at once apparent. Magnitude of the deviations differed from animal to animal. For example, 6 minutes after onset of perfusion, when mean esophageal temperature of all animals was 27°C, dog #1525 (Fig. 1-a) demonstrated a 2°C difference between right and left ventricular myocardium, whereas, in dog #1501 (Fig. 1-b) the difference was 11°C. After 12 to 15 minutes of cooling, when esophageal temperature of the animals was between 17 and 22°C, a critical temperature in so far as onset of fibrillation is concerned, temperature differences were more pronounced. A correlation was noted between decrease in heart rate and increased temperature gradient. As cooling continued to 10°C (esophageal) remarkable temperature variations were noted. In dog #1501 (Fig. 1-b), just prior to rewarming the right auricular myocardium was 14°C, while the left ventricular myocardium was 5.2°C.

With onset of rewarming, most animals promptly demonstrated a decrease in temperature variation, but in some, as rewarming proceeded, increased gradiations again became more apparent (Fig. 1-b). Probes from locations which manifested the greatest and most rapid fall in temperature during cooling revealed the reverse upon rewarming.

As cooling progressed, esophageal temperatures in most animals less closely approximated heart temperatures. The examples in Fig. 1 demonstrate that at 10°C the latter was ± 5°C of the esophageal readings. During rewarming the same discrepancy was observed.

Of the 14 animals in this study, none fibrillated during the cooling phase; 2 (Fig. 2-A and B) did during rewarming. No characteristic difference in temperature pattern, however, could be ascertained in the latter



A B

FIG. 2. Cardiac temperature gradients in 2 animals subjected to profound hypothermia and rewarming with fibrillation during rewarming.

dogs from that observed in those which did not fibrillate.

Discussion and conclusion. It is apparent from these studies that induction of hypothermia by extracorporeal perfusion and direct blood cooling results in marked gradients of cardiac temperature. In view of the fact that not a single animal fibrillated during cooling, it would seem that either (1) such gradients *per se* are not a factor in invoking fibrillation, or (2) the gradients must be of greater magnitude than those observed. During rewarming, when fibrillation did occur in 2 instances, temperature patterns were no different from those of animals without arrythmias.

An obvious explanation for the usual temperature gradients encountered and the variation between animals rests on the premise that there is a difference in blood flow through the various portions of the heart. This in turn may be related to catheter position, pump flow rates and other as yet unknown hemodynamic changes. For example, the precise location of the outflow catheters could well determine volume of blood available for circulation through the pulmonary vascular bed. As a consequence of such flow differences, it is also probable that oxygen gradients are likewise present. In view of the possibility that such a situation disposes to fibrillation(4), further study of this facet is contemplated.

Summary. During the production and reversion of profound hypothermia with extracorporeal perfusion temperatures were recorded from various sites throughout the heart. In all animals studied thermal gradients were observed. In some instances during cooling temperature variations as marked as 10°C were recorded. During rewarming similar differences were noted. From these studies it is concluded that thermal gradients alone are not responsible for hypothermic ventricular fibrillation.

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Fibrinolytic Activity in Fluid from Gingival Crevice.* (26308)

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Recent investigations have demonstrated the presence of fibrinolytic systems in various human body fluids(1,2,3). These systems resemble the fibrinolytic system in the blood but differ in that normally no or only very small amounts of plasminogen and inhibitory agents are present(4,5,6,7). Albrechtsen and Thaysen(8) found the fibrinolytic sys-

* This investigation was supported by grants from Swedish Med. Research Council.

tem in parotid and mixed oral saliva to contain small and variable amounts of plasminogen activator and large amounts of a proactivator of plasminogen. Like other body fluids, the saliva contained no plasminogen or inhibitory agents. They thought the plasminogen activator in the parotid saliva might help to resolve any fibrin clots formed in the Ductus Parotideus Stenonii. This investigation appears to be the only one published on the fibrinolytic system of fluids and tissues of the oral cavity.

During the last few years the tissue fluid passage through the gingival crevice has been considered in the etiology of peridontal diseases(9,10).

Discussing the histopathology of peridontal diseases Box(11) stated that the pocket is in reality a capillary space. The intimate relation between crevice and vascular system has been verified by Brill and Krasse(12). Intravenously injected fluorescein sodium was recovered from the crevice of clinically healthy gingivae of dogs in a very short time. The crevice thus seems to take part in the extravascular circulation, and investigation of the plasma constituents of the crevicular fluid appears worth while.

In our investigation we studied fluid from crevices of clinically healthy gingiva for its content of fibrinogen and fibrinolytic factors: plasmin, plasminogen activator, proactivator of plasminogen and plasminogen.

Methods. Teeth and gingivae were carefully dried. The tips of triangles (20 mm^2) of filter paper Munktell nr 3 were then cautiously placed for 3 minutes into the interproximal part of one crevice, care being taken to avoid contamination of the sample with saliva. Pilot studies had shown this time to be sufficient for filter paper to become saturated with tissue fluid. It was possible to repeat this procedure at intervals of 15 minutes without loss of fibrinolytic activity.

Fibrinolytic activity was determined on unheated and heated bovine Astrup fibrin plates as described by Nilsson *et al.*(13). The saturated filter papers were placed immediately on the fibrin plate. The methods described worked with a precision of about ± 15%. Proactivator and plasminogen activity were determined on heated bovine plates after addition of streptokinase or urokinase to the samples and principally as described previously(14).

TABLE I. Fibrinolytic Activity of Tissue Fluid from Gingival Crevices of 11 Females (19-21 Years Old) with Clinically Healthy Gingiva.*

Subjects	Heated plates (mm^2)	Unheated plates (mm^2)
1	104	142
2	105	167
3	52	180
4	50	162
5	62	136
6	88	159
7	120	157
8	47	175
9	95	143
10	76	203
11	134	148

* Activity on the plates was recorded as the diameter product in mm^2 of the lysed zone after 20 hr incubation at 37°C (avg of 3 determinations).

Eluates of the saturated filter papers were tested for fibrinogen as judged by their capacity to react with rabbit antibodies to human fibrinogen (Latex-Anti-Human-Fibrinogen-Reagent, Hyland Labs., Los Angeles).

Results. It is clear from Table I that the tissue fluid possessed plasmin activity. Plasmin activity of the crevicular fluid corresponded approximately to a trypsin solution of 15 μg of trypsin per ml. The lytic area obtained on the unheated plates was considerably larger than that on the heated plates, indicating that the fluid also contained plasminogen activator. After addition of streptokinase or urokinase to the tissue fluid activity on the heated plates increased to approximately the double value indicating presence of plasminogen as well as of proactivator.

After heating at 80°C at pH 2 for 45 minutes the crevicular fluid showed no activity on heated plates while activity on unheated plates remained unchanged. The activator is thus of the thermostabile type.

No plasmin or activator could be demonstrated in the saliva (Gustafsson and Nilsson, to be published) or in samples from other areas of the oral mucosa, not even on the outer surface of the marginal gingiva. Dental plaques on the tooth surface also seemed to

be devoid of plasmin and activator activity.

The activator of the crevicular tissue fluid was inhibited by ε-amino caproic acid and by δ-amino valeric acid which are known to be potent inhibitors of plasminogen activation (15), and not normally to occur in the organism. However, the latter amino acid has been demonstrated in saliva from patients with periodontitis, presumably as a product of local bacterial putrification(16).

When the crevicular fluid was tested against rabbit antibodies to human fibrinogen a positive reaction was obtained indicating presence of the homologous antigen. In the saliva no fibrinogen could be demonstrated by the same test.

It may be objected that the activity on the plates was due not to activator and plasmin but to bacterial proteases. Crude cultures of oral bacteria from the margin of clinically healthy gingiva and from the eroded marginal gingiva of patients with necrotizing gingivitis were added to heated and unheated fibrin plates. Neither of these cultures produced any lysed area. We also collected bacterial samples from the crevice after disinfecting the gingiva with an iodine-glycerine solution(17) before and after insertion of the filter papers. In a few cases culture of samples collected after such disinfection gave no growth indicating absence of viable bacteria. The fibrinolytic activity of such samples did not differ from that of samples giving growth.

Discussion. These last findings and actual existence of the precursor of the fibrinolytic enzyme as well as of the activator in the crevicular tissue fluid indicate that the fibrinolysis noted is attributable to presence of plasmin and activator in the crevicular fluid. The fact that ε-amino caproic acid and δ-amino valeric acid inhibited the activity on the unheated plates may be taken as support for presence of a plasminogen activator and plasminogen. These amino acids do not inhibit the fibrinolytic effect of trypsin and plasmin.

In the discussion on the pathogenesis of peridontal diseases the state of the ground substance has recently been considered, especially the acid mucopolysaccharides and the carbohydrases(18). A major part, however, of the water soluble extractives of connective tissue consists of plasma proteins(19). Our finding of plasmin and plasminogen activator in the crevicular tissue fluid appears also to merit consideration in evaluation of the state and function of the connective tissue of the gingiva. The fibrinolytic factors in the crevicular fluid might be of significance in counteracting the deposition of fibrin and other proteins at the junction between the gingival epithelium and the tooth. Our finding that at least traces of fibrinogen occur even in clinically healthy gingivae argues for a possible formation of fibrin there. In preliminary experiments on patients with necrotizing gingivitis we have found a decrease in fibrinolytic activity in the crevicular fluid at an early stage of the disease, despite the increased flow of tissue fluid in pockets of inflamed gingivae(20).

Summary. The crevicular fluid of 11 female subjects was studied for its content of fibrinolytic factors. It was found to contain plasmin, plasminogen activator, proactivator and plasminogen. The activator activity was inhibited by ε-amino caproic acid and δ-amino valeric acid. Fibrinogen was demonstrated in the crevicular fluid by the reaction of the latter to rabbit antibodies to human fibrinogen. The only one of these factors to be found in the saliva was the proactivator. The significance of an active fibrinolytic system in the crevicular fluid in etiology of peridontal diseases is discussed.

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Supplementary Role of Hydralazine in Reversal of Endotoxin Shock with Metaraminol and Hydrocortisone. (26309)

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Endotoxin shock in the dog can be reversed with a combination of a pressor agent and hydrocortisone, but there still remains the problem of decreased renal function due to reduced renal blood flow(1,2). Because hydralazine has been shown to increase renal blood flow(3-6), the following experiments were designed to measure the additive effect of this agent with metaraminol and hydrocortisone in reversal of canine endotoxin shock.

Methods. Four groups of adult male mongrel dogs, anesthetized with Na Pentobarbital (30 mg/kg), were injected intravenously with a standardized lethal dose of *Escherichia coli* endotoxin, as previously described(1). Measurements of blood pressure and urine output were made continuously for 9 hours, along with hourly determinations of hematocrit and arterial blood pH. Hydralazine,* metaraminol,† and hydrocortisone‡ were administered alone or in combination at that time after endotoxin administration when the animal was manifesting shock with a blood pressure of less than 70 mm Hg systolic, and anuria or oliguria of less than 4 ml/hr.

Group 1 consisted of 12 control dogs given 0.55 mg/kg of endotoxin intravenously. Group 2 involved 6 dogs given the same dose of endotoxin but treated with varying doses of hydralazine according to the output of urine measured at 30 minute intervals. Group 3 included 10 dogs given the lethal dose of endotoxin and then treated with metaraminol and hydrocortisone. Groups of control animals were not treated with either metaraminol or hydrocortisone alone, since previous studies had shown that neither agent reversed endotoxin shock(1). The fourth group of 10 dogs injected with the lethal dose of endotoxin were given hydralazine, and a combination of hydrocortisone and metaraminol in amounts identical to Group 3. That is, one hundred mg of hydrocortisone was infused intravenously, followed by a 1% solution of metaraminol in amounts that maintained systolic blood pressure between 90 and 100 mm of Hg. Hydralazine was administered at varying periods of time after injection of endotoxin.

To clarify the time-dose relationship of hydralazine in the fourth group of animals, it is necessary to review briefly the hemodynamic effects of endotoxin in the dog. Immediately following injection of endotoxin a

* Supplied as hydralazine HCl by Research Dept., Ciba Pharmaceutical Products, Inc., Summit, N. J.

† Supplied as Aramine Bitartrate 10% by Research Division, Merck, Sharp and Dohme, Philadelphia, Pa.

‡ Supplied as Solu-Cortef by Upjohn Co., Kalamazoo, Mich.

precipitous drop in systolic blood pressure occurs, due in large part to the hepatosplanchnic pooling of blood(7). Either of 2 characteristic hemodynamic patterns follow this initial hypotensive period, and each were observed in the present study. The first type of response is recovery of the blood pressure to pre-endotoxin levels, and is associated with oliguria or anuria. When this pattern was present, hydralazine was injected in order to correct the flow of urine, before hydrocortisone and metaraminol were administered for sustaining the blood pressure. The second pattern of response differs from the first in that the initial hypotension is prolonged, and in such instances hydrocortisone and metaraminol were administered first in order to support the blood pressure, followed by administration of hydralazine.

Results. None of 12 control dogs given endotoxin survived beyond 24 hrs. The mean hourly measurements are incorporated in Fig. 1. The fulminating course of these animals was marked by hypotension, severe oliguria, hemoconcentration, acidosis, and output of an acid urine.

Although none of the 6 animals in Group 2 treated with hydralazine survived, 2 findings were in contrast to those in the control group. The characteristic acidosis did not appear, and output of urine was consistently greater, although the hypotension persisted. Data on this group are presented in Table I, showing blood pressure and output of urine in relation to administered dose of hydralazine.

Six of the 10 animals in Group 3 treated with the combination of hydrocortisone and metaraminol survived. On the other hand, 8 of 10 animals in Group 4 survived when given hydralazine in addition to hydrocortisone and metaraminol. Measurements in this group of animals are shown in Table II. Eight of the 10 animals were given hydralazine before hydrocortisone and metaraminol, that is, there was a tendency for the blood pressure to approach normal after the initial decline but oliguria was severe. Two deaths occurred in this group. Two animals received hydralazine after steroid-pressor

TABLE I. Systolic Blood Pressure and Urine Output in 6 Dogs Given a Lethal Dose of *E. coli* Endotoxin and Treated with Hydralazine. Determinations recorded for control period of 1 hr and then hourly post-endotoxin.

Time of observation	Dog No. and wt (kg)																Mean values		
	1 12.4			2 11.6			3 10.4			4 8.7			5 13.7			6 13.2			
	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.
Control	170	6.4		200	9.1		205	10.5		185	10.3		160	16.4		115	.6		172	8.9	0
15 min. post-endo.	55			65			50			60			70			45					0
1 hr *idem*	140	12.5		180	7.9		65	5.6		150	3.0	.9	70	.0	3.0	50	.1		109	4.9	2.0
2 "	130	3.3	.6	80	.2	3.0	50	.0	3.0	125	7.2	6.0	70	2.0		45	.0	1.4	83	2.1	2.8
3 "	80	.2	1.8	120	1.0	3.0	60	.0	3.0	130	14.7		75	.5	2.4	65	.0	2.8	88	2.7	2.4
4 "	82	.1	2.0	150	.0	4.3	75	.0		135	16.5		130	8.2		115	.0	3.0	114	4.1	1.8
5 "	95	.2	1.2	160	.0		65	.0	3.0	145	13.8		125	.6		143	10.0		122	4.1	2.1
7 "	65	.4	4.2	125	.2	1.3	55	.0		135	12.0		115	.0		155	13.2		108	4.2	2.7
9 "	90	.0		95	.0		85	.2	2.2	110	7.6		160	.0		150	11.7		117	2.0	2.2
Total hydralazine (mg)			9.8			11.6			11.2			6.9			5.4			7.2			8.7
Survival post-endotoxin	18.0 hr			18.0 hr			18.5 hr			23 hr			24 hr			18.5 hr					

BP = Systolic blood pressure; UO = Urinary output in ml; Hyd. = Hydralazine in mg.

TABLE II. Systolic Blood Pressure and Urine Output in 10 Dogs Given a Lethal Dose of *E. coli* Endotoxin and Treated with Metaraminol, Hydrocortisone, and Hydralazine. Determinations recorded for a control period of 1 hr and hourly after endotoxin.

	Dog No. and wt (kg)																	
	1* 11.0			2 9.0			3* 10.0			4* 11.3			5* 11.3					
Time of observation	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.			
Control	210	8.6		202	11.1		164	10.8		125	9.4		170	9.8				
15 min. post-endo.	55			70			40			35			65					
1 hr *idem*	95	2.2	.9	155	8.3	.6	95	19.4	.9	65	.0		55	15.9	.9			
2 " "	95	10.8		85	14.6		110	20.8	.9	85	1.2	.4	115	2.3				
3 " "	110	.8		100	8.5		135	32.4		130	10.0		105	.5				
4 " "	125	9.5		145	22.1		145	36.0		165	16.6		125	.2	.4			
5 " "	150	11.6		140	49.0		140	51.8		165	19.2		135	.0	1.2			
7 " "	125	13.7		130	19.9		120	66.2		125	20.4		140	13.8	1.6			
9 " "	130	10.1		140	40.2		120	38.5		125	22.6		135	8.4				
Total hydralazine (mg)			.9			.6			1.8			.4			4.5			
Survival post-endotoxin	Permanent			Permanent			Permanent			Permanent			Permanent					

	Dog No. and wt (kg)															Mean values (dogs 1-10)		
	6* 12.0			7* 9.6			8* 10.0			9* 10.0			10* 15.0					
Time of observation	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.	BP	UO	Hyd.
Control	200	8.2		225	11.6		180	10.2		142	10.5		145	10.6		176	10.1	0
15 min. post-endo.	45			60			50			50			45			52		0
1 hr *idem*	135	2.0	.9	140	12.8	.6	90	1.9	.9	75	4.1	.9	185	14.1	1.2	109	8.1	.78
2 " "	80	1.2	1.0	135	15.8		95	.1	.6	93	.3	1.8	160	26.2		105	9.3	.47
3 " "	105	2.3	.9	130	14.0		108	9.5	.9	115	.7		162	27.3		120	10.6	.18
4 " "	165	1.7		135	18.0		125	4.0	.5	135	7.8		145	19.4		141	13.5	.09
5 " "	185	21.9		155	10.4		145	11.3		130	12.0		140	15.0		148	19.0	.12
7 " "	180	40.0		150	12.5		165	9.8		125	13.0		135	13.0		137	21.0	.16
9 " "	150	40.3		160	9.3		165	10.1		130	18.6		140	12.8		139	23.0	0
Total hydralazine (mg)			2.8			.6			2.9			2.7			1.2			
Survival post-endotoxin	Permanent			Permanent			18 hr			16 hr			Permanent					

* Received hydralazine first.

BP = Systolic blood pressure; UO = Urinary output in ml; Hyd. = Hydralazine in mg.

therapy, and both survived. Mean average hourly determinations of this group receiving all 3 agents are compared (Fig. 1) with the untreated control animals, those receiving only hydralazine, and those animals receiving only hydrocortisone and metaraminol. The most significant feature in the hydralazine-hydrocortisone-metaraminol treated animals was the marked volume of urine recorded in comparison to the other groups.

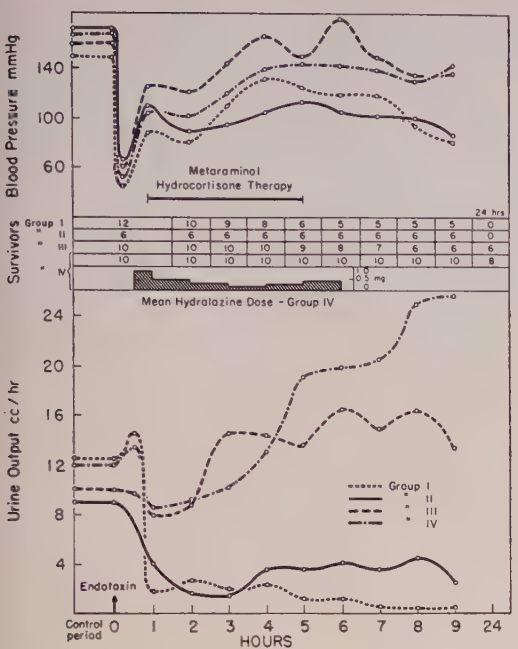

FIG. 1. Time-dose relationship of 4 groups of dogs given a lethal dose of endotoxin. Group 1 = untreated controls; group 2 = treated with hydralazine alone; group 3 = treated with hydrocortisone and metaraminol; group 4 = treated with hydrocortisone, metaraminol and hydralazine. Curves illustrate avg hourly blood pressure and output of urine. Animals surviving 24 hr after endotoxin are indicated for each group.

Discussion. In studies on the pathogenesis of canine endotoxin shock attempts have been made to reverse the peripheral vascular collapse with various agents. After onset of hypotension, and oliguria or anuria, the most consistently successful results obtained in this laboratory have followed administration of a combination of hydrocortisone and metaraminol. The present study has demonstrated that hydralazine will increase urine flow in dogs having severe hypotension. This is in contrast to the untreated control series of animals that had a higher blood pressure but a consistently lower output of urine. When the systemic pressure was supported with a pressor drug and steroid, hydralazine had a favorable effect on output of urine, even though the blood pressure was lower than in the pressor-steroid treated group. These results are of interest since hydralazine is usually employed as an anti-hypertensive drug.

Summary. Canine endotoxin shock is characterized in part by hypotension, oliguria, hemoconcentration and acidosis. The "shock" can be reversed in some animals with a combination of a pressor drug and hydrocortisone. The addition of hydralazine to this combination greatly augments the flow of urine, and possibly contributes to the survival of animals.

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Presence of Two Glutamic-Oxalacetic Transaminases in Serum of Dogs Following Acute Injury of the Liver.* (26310)

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We have reported recently that human and animal tissues such as heart and liver contain 2 glutamic-oxalacetic transaminases

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(1). At pH 7 the previously known enzyme (GOT I) has anionic properties, whereas the newly discovered transaminase (GOT II) acts as a cation. Separation, therefore, can be made easily by electrophoresis or by use of ion exchangers(2). The relative proportion of the 2 enzymes also can be determined indirectly by a differential test. In most tissues investigated the contribution of GOT II to total activity has been found to vary between 40 and 70%. In normal human serum the presence of GOT II has not been established with certainty, and in a few serums with high transaminase activity due either to recent myocardial infarction or to acute viral hepatitis the proportion of GOT II was found to be small. It was thought to be of interest to determine in dogs acutely poisoned with carbon tetrachloride whether the high serum activity of GOT resulting from this injury(3) might contain at any time substantial proportions of the new transaminase. Because activity of enzymes in serum must also depend on rate of elimination, we have studied in dogs the disappearance curves of intravenously injected, highly purified preparations of the 2 GOT's as well as of GPT (glutamic-pyruvic transaminase).

Methods. Production of hepatic injury. Four healthy dogs received carbon tetrachloride by gastric intubation in doses of 2 ml per kilo of body weight. Blood specimens were taken from the external jugular vein, irrespective of food intake, twice or three times before the beginning of the experiment, to serve as controls, and over a period of 12 days following administration of the toxic agent. The blood was allowed to clot and the serum was separated by centrifugation.

Determination of enzymatic activities. Determination of transaminase activities was by the differential spectrophotometric test at 38°C, as previously described(1). Activities are expressed as micromoles of oxalacetic acid formed per hour per milliliter of serum (μM/hr/ml). The differential test consists of 2 determinations, one at pH 7.4 in presence of high concentration of aspartate (10^{-1} moles/liter) to determine the sum of activities of GOT I and GOT II, the other at pH 6.0 in presence of low concentration of aspartate (5×10^{-3} moles/liter) to determine almost exclusively GOT II. Corrections for the slight nonenzymatic destruction of DPNH (reduced diphosphopyridine nucleotide) were applied in each case; in our experience these amount to 1.5 and 2.2 density units per minute at pH 7.4 and 6.0, respectively. Activity quotients (activity at pH 6.0 divided by activity at pH 7.4) for our purest preparations from dog liver were 0.544 and 0.027, respectively, for GOT II and GOT I. From the activity quotient determined in a serum with an unknown mixture of the 2 transaminases the proportion of each activity can be calculated with a fair degree of accuracy.

Preparation of purified transaminases and intravenous administration to dogs. The method of separation and purification of the 3 transaminases, GOT I, GOT II and GPT, from dog liver was presented by one of us (2) at a recent meeting and will be the subject of a forthcoming paper. The preparations used for intravenous injection were purified several hundredfold. Specific activities based on milligram protein nitrogen were 35,000, 60,000 and 20,000, respectively, for GOT I, GOT II and GPT. The preparations were injected in doses of about 15,000 μM/hr per kilo of body weight, contained in 0.3 ml of solution. Serial blood specimens were taken until serum activity had returned to normal values.

Results and discussion. Carbon tetrachloride poisoning. In confirmation of earlier tests(3), maximal total GOT activity was observed in all 4 dogs 48 hours after administration of carbon tetrachloride. Activities then ranged from 448 to 995 μM/hr/ml as compared with 0.89 to 1.40 for control specimens. By the ninth day values had returned almost to normal. The findings in one dog are summarized in the Table, while individual activities of GOT I and GOT II, calculated from the activity quotients, for the 4 dogs, are plotted in Fig. 1. For the periods between 16 and 96 hours it is seen that at the time of maximal total activity both enzymes exhibited peaks. Activity quotients then ranged from 0.10 to 0.11, an indication that GOT II contributed 14 to 16% (mean

TABLE I. Distribution of Serum Glutamic-Oxalacetic Transaminases Following Administration of Carbon Tetrachloride in a Dog.

Hr after CCl_4	GOT, μM/hr/ml pH 7.4	pH 6.0	Quotient	GOT II %	μM/hr /ml
Control*	1.04	.052	.050		0
"	1.22	.030	.025		
"	1.33	.017	.013		
16	44.0	1.13	.026	0	0
18	41.6	1.15	.028	.2	.1
20	43.2	1.85	.043	3.1	1.3
24	54.0	2.19	.041	2.7	1.4
40	760.0	68.3	.090	12.2	92.8
48	824.0	82.4	.100	14.1	116.2
64	139.4	7.1	.051	4.7	6.6
72	47.5	3.06	.064	7.2	3.4
88	11.3	.74	.065	7.6	.9
96	8.5	.62	.073	8.9	.8
112	6.5	.37	.057	5.8	.4
168	3.9	.23	.059	6.4	.2
192	3.3	.19	.058	6.0	.2
216	1.6				
240	2.2				
264	1.8				
288	1.4				

* 4, 2 and 0 days before inj. of CCl_4.

14.7) to total activity. At 40 hours the distribution was quite similar, while at 24 and 64 hours, GOT II averaged 3.5 and 4.5%, respectively, of total activity. Whether, in normal dog serum, GOT II is present at all is difficult to decide, since its activity at pH 6.0 is extremely small and the accuracy of the test, therefore, low. Mean quotient with standard error of the mean in 9 control serums was 0.029 ± 0.006, which is nearly the same as the quotient of purified GOT I (0.027). Interestingly enough, in rat serum we have found that GOT II contributes 20 to 30% to total activity.

It may be of interest to recall the findings on GPT following administration of carbon tetrachloride to dogs(3). Peak activity generally occurred 1 day later than did that of GOT, and was also somewhat higher. GPT activity also decreased more slowly than did that of GOT, with the result that the activity-time curve of GPT was very much broader than that of GOT. On the thirteenth day after administration of the hepatotoxic agent, GPT activity was still 16 to 20 times control values.

Intravenous administration of transaminases. Disappearance rates of exogenous GOT I, GOT II and GPT in normal dogs are shown in Fig. 2. The 3 enzymatic preparations were also injected into groups of 3 additional dogs and the decline of their activities followed. In each instance the results were quite similar to the ones illustrated. GOT II disappeared very rapidly from the blood stream, and 6 to 8 hours after injection usually none of the injected enzymatic activity remained. A much slower elimination was the rule for GOT I. Here enzymatic activity did not return to control levels until 3 to 4 days after injection. The slowest rates were observed for injected GPT, which was present in blood for as long as 18 days.

It seems that the differences in elimination curves of exogenous GOT I, GOT II and GPT bear a relationship to the activity curves in serum of endogenous transaminases following poisoning of the liver by carbon tetrachloride. GOT II is eliminated so rapidly that it cannot build up as high a concentration in blood as can GOT I or GPT. GPT, on the other hand, disappears so slowly that it shows a numerically greater maximal

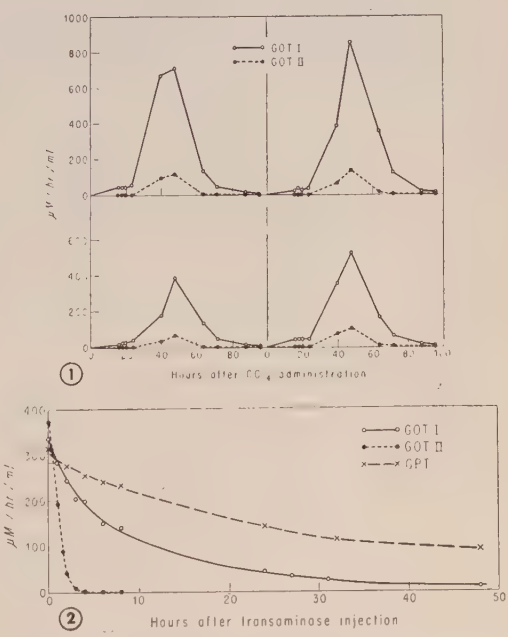

FIG. 1. Serum activities of GOT I and GOT II following carbon tetrachloride poisoning in 4 dogs.
FIG. 2. Serum transaminase activities following intrav. administration of purified transaminase preparations in 3 dogs.

activity than does GOT I and remains elevated for a considerably longer time after hepatic injury. Similar observations with regard to (total) GOT and GPT also have been made in patients with acute viral hepatitis or jaundice due to chlorpromazine(4-8), but a detailed study of the distribution of GOT I and GOT II in these conditions has not been made.

Another reason for the very low levels of serum GOT II in carbon tetrachloride poisoning may be sought in the recently discovered fact(9) that this enzyme is bound within the mitochondrion, in contrast to GOT I and GPT which appear to be present exclusively in the soluble part of the cell. While GOT II is readily released from its confinement inside the mitochondrion in the preparation of aqueous homogenates, we have noted that the enzyme is quite tightly bound in mitochondria isolated by standard procedure, and is then difficult to solubilize. It is conceivable that also under physiologic and pathologic conditions this enzyme may be released with some difficulty.

Summary. Serums of dogs poisoned with carbon tetrachloride exhibited high activities of GOT I and GOT II with maxima 48 hours after administration of the toxic agent. At no time, however, did GOT II contribute to total activity more than 16%. In control serums the presence of GOT II could not be established with certainty. Disappearance rates of intravenously administered purified preparations of transaminase also were studied in dogs. Activity of GOT II decreased very much faster than that of GOT I. This difference may explain the uneven distribution of the 2 serum transaminases in carbon tetrachloride poisoning.

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Glycogen Content of Normal Lymphocytes.* (26311)

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The glycogen content of the formed elements of the blood is said to reside in the leukocytes, red cells and platelets containing little or none of this polysaccharide(1). Furthermore, it has been assumed that the major portion of that present in the white blood cells is contained in the myeloid elements. These assumptions are based on histochemical examination of the blood from normal individuals and leukemic patients(2,3) and from chemical determinations on the leukocytes from patients with acute and chronic lymphocytic leukemia(4,5). Glycogen assays of the lymphocytes found in these states have revealed extremely low levels. It has therefore been deduced that the lymphocyte of the normal individual contains a meager amount of this polysaccharide. However, direct measurements of normal lymphocytes have not been possible because of the difficulty of separating these cells from the other formed elements of the blood except when they are present in elevated and ex-

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tremely pure numbers. Recently a method has been devised for isolating lymphocytes from the total white cell population of normal individuals thus making possible determination of their glycogen content(6). The results will be described below.

Material and methods. Venous blood was collected in heparinized tubes from normal adults with normal peripheral leukocyte counts and differentials. For obtaining all white cell elements whole blood was allowed to settle for 30 minutes in a freshly prepared saline solution of fibrinogen. The supernatant leukocyte-rich plasma was then withdrawn. The lymphocytes were obtained from whole blood by the method of Jago in a refrigerated centrifuge at 4°C, and total cell yield was determined by white cell count. The percentage purity was determined by differential counts done on 100 white cells from smears made from this lymphocyte rich plasma.

The leukocyte and lymphocyte-rich plasma was then centrifuged at 3000 rpm for 15 minutes and supernatant cell free plasma was decanted. The button was treated with 2 ml of boiling KOH for 30 minutes. Glycogen was precipitated by adding 2 cc of 95% ethanol to the KOH solution and allowed to stand for a minimum of 3 hours at 37°C.

After precipitation, the tubes were centrifuged at 3000 rpm for 15 minutes. The clear supernatant fluid was gently decanted from the packed glycogen and the tubes allowed to drain in an inverted position. The glycogen was dissolved in 2 ml of distilled water and was determined by the anthrone method using distilled water as a blank and a glucose standard in a Beckman DU Spectrophotometer at 620 mμ.

Results. The glycogen content of total white cells from the blood of 10 normal individuals was determined. Mean leukocyte glycogen per 10^{10} total leukocytes was 50.9 mg with a range of 41.2 to 56.6 (Table I). These results are in close agreement with those of Valentine, Follette and Lawrence (3) for mean total leukocyte glycogen using a similar technic.

Glycogen content of normal lymphocytes alone was determined. A minimum of 10^4 cells containing at least 90% lymphocytes was used for each test. The mean of 20 determinations on normal adults was 113.67 mg/10^{10} cells. In 5 instances the determinations were performed in duplicate. The difference between paired analysis expressed as percent of their average value did not exceed 2.75%. The complete results are shown in Table I.

TABLE I. Glycogen Content of Leukocytes.

	No. deter.	Mean mg per 10^{10} cells	Range	S.D.
Total leukocytes	10	50.9	41.2- 56.6	5.85
Total lymphocytes	20	113.67	65.0-189.8	29.79

To confirm that the reducing substance obtained after precipitation with alcohol was glycogen, it was treated with a 1:10 dilution of saliva in saline for one hour at 37°C at pH 6.5. An equivalent portion of alcohol precipitate was treated with saline for a similar period. Both samples were then boiled with one ml of KOH and reprecipitated with ethyl alcohol. The glycogen remaining was determined by the anthrone method. In 2 experiments it was found that approximately 80% of the carbohydrate was digested.

Discussion. It has been shown that a considerable amount of glycogen is present in normal human lymphocytes. We have determined that lymphocytes comprise 40% of the cellular population of "total cells" as obtained by the fibrinogen separation method. On the basis of this and the glycogen values mentioned above it is apparent that the lymphocyte contributes the major portion found in "total cell" value. This finding is in contradiction to the previous assumption of low lymphocyte glycogen content based primarily upon histochemical examination of blood smears after periodic acid-Schiff staining. The visual method reveals that all of the mature granulocytes contain PAS positive material in the cytoplasm whereas only a few of the lymphocytes present contain granules of glycogen. The chemical determination described above casts doubt on conclusions as to quantity of glycogen present in the lymphocytes determined by the PAS method. The reasons for the variances be-

tween these two methods are being investigated.

The present findings emphasize the difference between normal lymphocytes, which are relatively rich in glycogen, and leukemic lymphocytes and lymphoblasts which contain little of this carbohydrate. Unfortunately, until recently this was the only practical source of large numbers of cells of pure yield. By adoption of the above described method satisfactory numbers of lymphocytes can be separated from the blood of normal individuals. Glycogen determinations on these cells reveal appreciably different results than those found in the above mentioned malignant conditions. These findings emphasize the danger of drawing conclusions concerning metabolic activity and chemical constitution of normal cells from observations of abnormal cells.

Summary and conclusion. Lymphocytes were isolated from the blood of normal individuals. Glycogen content of these cells was determined by the anthrone technic. A significant amount of this polysaccharide was found in these cells suggesting that normal lymphocytes contain more of this substance than the granulocyte. These findings are in contradiction to observations made on these cells by PAS method and assumptions based on chemical glycogen determinations of leukemic lymphocytes.

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Incorporation of Manganese into Duck Erythrocytes.* (26312)

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The appearance of intravenously-administered, labeled manganese in human erythrocytes follows a time-course like those for the hemoglobin precursors, iron and glycine. The administered manganese in erythrocytes, in both man and rabbit, is recoverable from the cells mainly in association with hemin, thus the provocative hypothesis has been advanced that the metal is present in the cells as a porphyrin complex(1). In the present work the erythrocytes of duck blood were found to take up labeled manganese reversibly *in vitro,* but no evidence for association of the metal with porphyrin was obtained. After x-irradiation of the animal, *in vitro* uptake decreased to a low level, which suggests that the uptake is a function mainly of reticulocytes. *In vivo,* intravenously-administered manganese appears to be taken up by erythrocytes during formation or maturation of the cells in bone marrow and also after the cells enter the circulation. The latter uptake likely reflects that observed *in vitro.* Some of the manganese entering the cells *in vivo,* presumably that taken up in marrow, was recovered in association with heme. This finding supports the hypothesis indicated above.

Experimental. Adult, male, Pekin ducks fed a mixture of commercial food pellets and corn were used. Blood was collected in heparin. The labeled manganese used, essentially carrier-free, divalent Mn^{54} in hydrochloric acid, was estimated by means of a well-type scintillation detector(2). The results and details of representative tests *in vitro* are given in Table I.

The packed-cell fraction of blood incubated with Mn^{54} contained an appreciable fraction of the metal, relatively little of which

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TABLE I. Uptake of Labeled Manganese by Duck Erythrocytes *In Vitro*. In each test, blood was mixed with .025 vol of a solution containing about 5×10^5 counts/min. of carrier-free, divalent Mn^{54} per ml of 0.05 N HCl. Except where indicated otherwise, the mixture was shaken at 37° under 5% CO_2 in O_2 for 2 hr and cells were collected and washed twice with 2.5 vol of 0.156 M NaCl in the cold. Cells and plasma plus washings were assayed for Mn^{54}. In Exp. 7, animal was exposed to 1000 r of X-rays (250 KVP, 1.9 mm Cu HVL, 85 r/min.) shortly after initial collection of blood.

Exp.	Fraction of Mn^{54} in cells	Treatment
1	.38	Cells not washed
	.35	Cells washed once with 4 vol of plasma at 25°
	.34	Cells washed twice with 4 vol of plasma at 25°
	.34	Cells washed twice with 4 vol of saline at 25°
2	.18	Buffy coat not removed
	.17	" " removed
3	.01	Cells collected immediately after mixing blood and Mn^{54}
	.16	Cells collected after incubating blood and Mn^{54}
4	.02	Blood plus Mn^{54} kept at 4°
	.16	" " " incubated
5	.15	Blood-Mn^{54} mixture incubated 2 hr
	.22	Blood-Mn^{54} mixture incubated 4 hr
6	.19	Cells collected after 2-hr incubation
	.11	Cells as above, but incubated 2 hr in unlabeled plasma
7	.13	Before irradiation of animal
	.16	20 hr post-irradiation
	.06	42 hr *idem*
	.03	64 hr "
	.02	164 hr "

was removed by washing the cells at room temperature with plasma or salt solution (cf Exp. 1). Removal of the buffy coat from packed, labeled cells after initial collection and after each of 2 washings with saline in the cold did not markedly decrease the amount of Mn^{54} associated with the cells (Exp. 2). Therefore, erythrocytes account for most of the manganese taken up by the blood cells. Cells collected immediately after mixing blood and manganese (Exp. 3) or from mixtures kept in the cold (Exp. 4) contained but a small fraction of the added metal. Thus, the uptake depends upon incubation and its duration (Exp. 5). Incubation of labeled cells with unlabeled plasma removed a large fraction of the Mn^{54} (Exp. 6). Consequently, the process involved in the uptake appears reversible. X-irradiation of the animal was followed by decrease in manganese uptake to a negligible level (Exp. 7); however, irradiation of blood *in vitro* had no effect on uptake. Iron uptake by the erythrocytes of duck blood *in vitro*, as the manganese uptake, decreases after irradiation of the animal, due to disappearance of reticulocytes from the blood(3). Thus, although other possibilities are not ruled out, it seems reasonable to ascribe the decrease in manganese uptake after irradiation of the animal to disappearance of reticulated erythrocytes from the blood.

Solutions of heme in acid ethyl acetate(4) obtained from mixtures of blood and Mn^{54} immediately and after incubation contained about the same fraction of the isotope (Table II, Exp. 1 and 2). Crystalline hemin preparations(5) obtained from cells of blood incubated with the isotope and from unlabeled cells mixed with isotope just before treatment for hemin isolation contained about the same fraction of isotope (Exp. 3 and 4). These results indicate no appreciable complex formation between cell porphyrin and manganese taken up *in vitro* and contrast with indications for such complex formation obtained in man and rabbit *in vivo*(1).

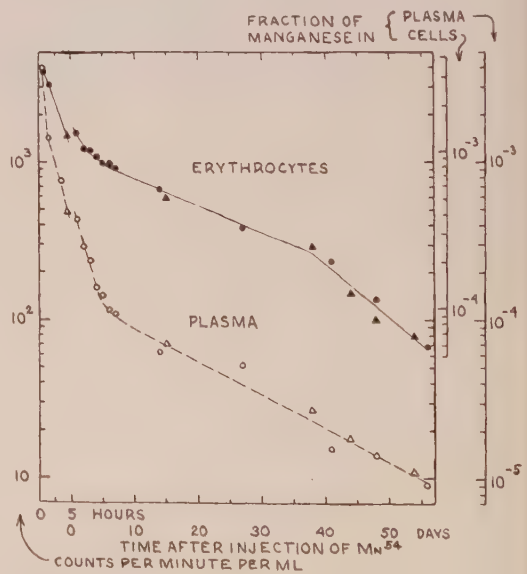
The distribution of intravenously administered Mn^{54} between erythrocytes, plasma, and excreta is illustrated in Fig. 1. The prompt appearance of the isotope in the cells and relatively rapid loss of isotope from the cells during the first few days of test period presumably reflects a reversible uptake by circulating erythrocytes like that observed *in vitro*. Since the lifetime of the erythrocytes is approximately 5.5 weeks (6-8), the marked loss of the manganese from the cells after about Day 40 appears referable to breakdown of cells labeled earlier. It seems likely that this early labeling occurred mainly in bone marrow rather in the circulation because the prompt initial uptake appears readily reversible and in man(1) incorporation of manganese into erythrocytes rather certainly

TABLE II. Mn^{54} in Heme and Hemin Preparations from Erythrocytes Labeled with Isotope *In Vitro* and *In Vivo*. *In vitro*-labeled erythrocytes were obtained by incubation of isotope with blood (Table I). *In vivo*-labeled cells were those of tests described in Fig. 1. Heme preparations (4) were made from whole blood, hemin preparations(5) from packed cells. Blood- or cell-isotope mixtures treated immediately for heme or hemin preparation respectively served as controls in *in vitro* tests.

Material from which heme or hemin was derived			Mn^{54} activity in or added to cells, counts/min./ml	Mn^{54} activity of: Cells / Blood	Heme / Cells	Hemin / Cells
Exp. 1.	Blood.	Not incubated	1.2×10^4	.01	.015	
		Incubated	2.6×10^5	.21	.010	
" 2.	"	Not incubated	2.0×10	.02	.013	
		Incubated	1.5×10^2	.15	.012	
" 3.	Cells.	Not incubated	2.5×10^5			.002
		Incubated	3.1×10^5	.25		.003
" 4.	"	Not incubated	1.8×10^2			.003
		Incubated	2.6×10^2	.18		.001
Animal 1	Blood, day 14		6.8×10^2	.92	.160	
	" " 56		6.8×10	.88	.220	
	Cells, " "		" × "	"		.045
Animal 2	Blood, " 0.2		4.5×10^3	.76	.020	
	Cells, " "		" × "	"		.003
	" " 15		1.8×10^3	.90		.029
	" " 48		3.0×10^2			.058
	Blood, " 54		2.4×10^2	.89	.150	

takes place during cell formation and/or maturation. Removal from the plasma was much faster, incorporation into cells much less, and early excretion was greater for the Mn^{54} than for labeled iron(6).

The following indicates that the state of some of the manganese that entered the cells *in vivo* differs from that obtaining *in vitro*: Hemolyzed cells(4) from blood obtained on Day 37 were dialyzed at room temperature against 250 volumes of stirred 0.156 *M* potassium chloride for 3.5 days, 0.005 *M* disodium ethylenediaminetetraacetate for one day, and 0.001 *M* manganese dichloride for one day. After dialysis the cell preparation contained 0.44 of the Mn^{54} originally present while hemolyzates from cells labeled *in vitro* and cells of Day 0.2 contained respectively 0.20 and 0.29 of the activity. The fractions of cell Mn^{54} found in heme or hemin preparations derived from cells collected after Day 0.2 were appreciably greater than found in the *in vitro* tests (Table II). The recovery of Day 0.2 was about the same as *in vitro*.



Results for this animal are indicated by circles. Animal 2, wt 3.10 kg, given 1.39×10^8 counts/min. /kg body wt. Results, indicated by triangles, have been adjusted to expectation for the dose of isotope given Animal 1. Initial distribution of Mn in plasma (87 ml blood/kg assumed, hematocrit of 0.46) or in extracellular fluid (250 ml/kg) corresponds theoretically to activities of 9.81×10^5 and 1.86×10^5 counts/min./ml respectively. Animal 1 excreted 0.12 of Mn by Day 1, 0.14 by Day 5, and less than 0.01 between Day 5 and 17. Curves were drawn by inspection.

FIG. 1. Uptake of Mn^{54} by duck erythrocytes *in vivo*. Animal 1 weighed 3.64 kg and was given 4.65×10^7 counts/min. of carrier-free, divalent Mn^{54} in 5 ml of .03 N HCl in .156 M NaCl/kg of body wt.

This indicates again that the early appearance of the isotope in the cells is attributable to uptake like that found *in vitro*. Although the fraction of the labeled manganese in the duck erythrocytes that was recovered in association with the iron-porphyrin complex is much smaller than found in man and rabbit(1), the results support the hypothesis that erythrocytes contain a manganese-porphyrin complex.

Summary. Duck erythrocytes *in vitro* take up labeled manganese added to blood. The uptake is mainly ascribable to reticulocytes. Following intravenous administration, the manganese appears promptly in the cells apparently as a function of the readily reversible uptake observed *in vitro* and as a function of incorporation in bone marrow. Manganese taken up by cells *in vivo*, but not *in vitro*, is recoverable to some extent in association with heme. This recovery is considered to support the hypothesis that erythrocyte manganese is present in part at least as a porphyrin complex.

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Isolation of a Thermolabile Serum Protein which Precipitates γ-Globulin Aggregates and Participates in Immune Hemolysis.* (26313)

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Recently, the authors(1) and Taranta(2) reported that normal human serum contains a heat-labile factor capable of precipitating soluble γ-globulin aggregates. Gamma-globulin aggregates have proved in the past a useful means for demonstration of rheumatoid factor, a high molecular weight, heat-stable serum protein possessing a strong affinity for γ-globulin and occurring predominantly in patients with rheumatoid arthritis (3). The difference in heat stability precluded the identity of the precipitating factor of normal serum with rheumatoid factor and strongly suggested its relation to the complement system. A relation to complement appeared particularly likely in view of the observations of several investigators(5,6, 7,8) indicating that complement is capable of reacting with γ-globulin aggregates and that it undergoes inactivation during this reaction.

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The present paper describes the method of isolation and some characteristics of the precipitating factor of normal serum. It also reports the finding that preparations of purified factor contained an activity distinct from the activity of any known complement component and which proved essential for immune hemolysis.

Materials and methods. Soluble γ-globulin aggregates were prepared according to a method similar to that described earlier(3,4). Three grams of Lederle F II γ-globulin, dissolved in 300 ml saline were heated at 63°C for 12 min. The aggregates were precipitated from this solution by addition of 20 g sodium sulphate. After one hour at 4°C the precipitate was collected, suspended in 20 ml distilled water, and dialyzed against 2 × 3 liter barbital buffer, pH 7.3. The resulting solution of γ-globulin aggregates contained 25-30 mg protein per ml. More than two-thirds represented material with an average s-rate of 90-110 S.

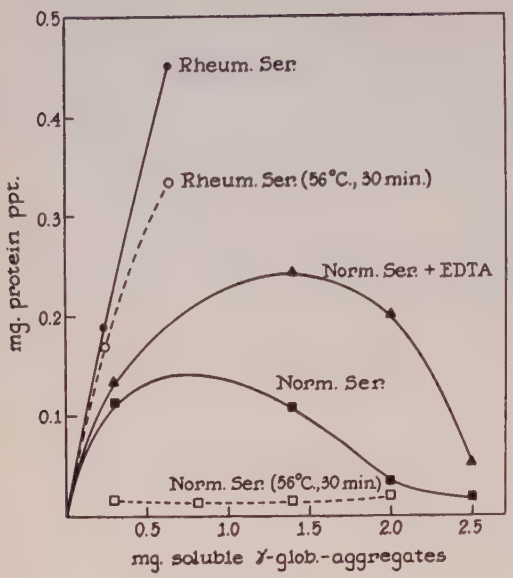

FIG. 1. Precipitin curves of normal human serum and of serum containing rheumatoid factor with soluble γ-globulin aggregates, showing the effect of heating and of EDTA upon precipitin reaction.

Preparation of 11 S component: 25-30 ml of a solution of γ-globulin aggregates were added to 200 ml fresh normal serum. Both parts contained 0.01 M Na_3EDTA. They were pre-cooled and after combination held at 4°C for 5 hours or overnight. The resulting precipitate was separated from the supernatant serum, and washed 3 times in 10 volumes phosphate buffer, pH 7, T/2 = 0.1. The washed precipitate was twice extracted in 6.5 ml cold phosphate buffer, pH 5.3, T/2 = 0.3. A thorough suspension was obtained by pipetting the precipitate for 5-10 minutes. The insoluble residue was then removed by centrifugation and the extracts filled into 6.5 ml lusteroid centrifuge tubes. After centrifugation at 114,000 × g in a Spinco rotor 40.3, for 40 min at 1°C, the supernatants of both extracts were combined and concentrated to 0.5 to one ml by ultrafiltration. The pH was adjusted to pH 7, using 0.2 M Na_2HPO_4.

Preparation of an R_{11S}: 3.5 ml of fresh serum containing 0.01 M Na_3EDTA were combined with 2.5 ml soluble γ-globulin aggregates containing 25-30 mg protein per ml and 0.01 M Na_3EDTA. One ml barbital buffer, pH 7.3, was also added. This mixture was held at 4°C overnight and centrifuged at 114,000 × g at 1°C for 1½ hours, using a Spinco rotor 40.3. The top 5 ml, which were recovered from the centrifuge tube, were diluted 1 → 2.5 to obtain a final dilution of 1 → 5 with respect to the original serum concentration, and were stored at 4°C for use within the subsequent 5 days. Ca^{++} and Mg^{++} were added to overcome the EDTA effect.

Quantitative precipitin reactions: To 0.2 ml portions of serum soluble γ-globulin aggregates were added in amounts ranging between 0.5 and 2.5 mg and final volume was adjusted to 0.4 ml with normal saline. After 15-20 hours at 4°C, amount of protein precipitated was determined by the Folin method.

Analytical ultracentrifugation was performed in a Spinco model E machine at 52,640 rpm at 20°C. For their analysis washed precipitates of serum, induced by soluble γ-globulin aggregates, were dissolved in glycine buffer, pH 3, T/2 = 0.1.

Results. Fig. 1 illustrates the precipitin reaction of normal human serum with soluble γ-globulin aggregates. For comparison the precipitin curve of a serum with rheumatoid factor activity was included. Although there was a striking quantitative difference between the reaction of rheumatoid serum and that of normal serum, the latter definitely precipitated soluble γ-globulin aggregates. While the activity of rheumatoid serum, as expected, was little diminished after heating at 56°C for 30 min, this treatment resulted in complete loss of activity of the normal serum. Depletion of Ca^{++} and Mg^{++} by EDTA on the other hand augmented the precipitation reaction of normal serum. More than 80 normal sera examined in this fashion were found to resemble each other, especially with respect to heat lability and augmentation of precipitating activity by EDTA. Regarding the extent of the reaction, considerable variation between different sera was encountered. Sera from children, especially umbilical cord serum, gave low precipitin curves. Three sera from agammaglobulinemia patients showed similarly low values, while some pathological sera, primar-

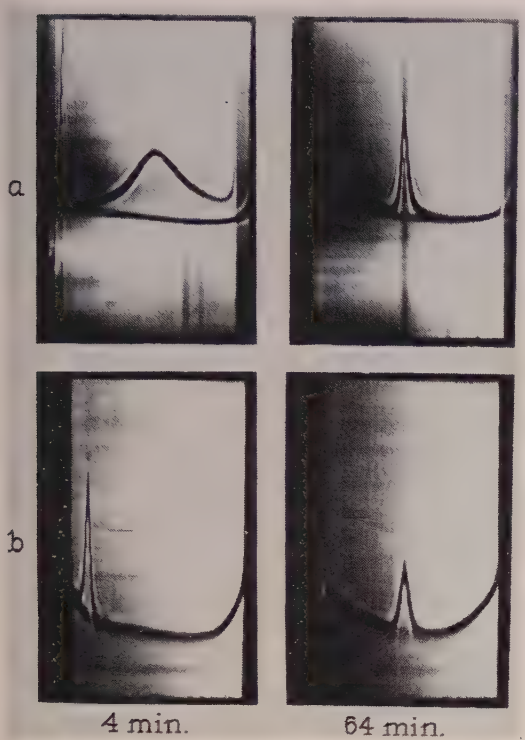

FIG. 2. Ultracentrifugal patterns (a) of a dissolved precipitate formed by normal human serum upon addition of soluble γ-globulin aggregates in presence of EDTA, (b) of the isolated precipitating factor (11 S component) of normal serum.

ily those from patients with hepatitis, exhibited a strikingly increased precipitating activity.

Precipitates of normal serum induced by soluble γ-globulin aggregates were dissolved in glycine buffer, pH 3, and analyzed in the ultracentrifuge. Invariably, 2 main components could be distinguished. One of these was heterogeneous and sedimented with an average s-rate of approximately 100 S, the other was homogeneous and sedimented much more slowly. The former represented γ-globulin aggregates and the latter an unknown constituent of serum. Variable amounts of 2 minor components with s-rates of 7 and 19 S were also seen. Fig. 2a depicts the ultracentrifugal pattern of a dissolved precipitate which was obtained from serum previously depleted of bivalent cations.

The main component derived from serum was extracted from precipitates with phosphate buffer, pH 5.3, ionic strength 0.3, at which conditions the aggregates remained largely insoluble. After additional purification which included density gradient ultracentrifugation, some highly homogeneous preparations were obtained consisting of more than 95% one component. The ultracentrifuge pattern of one preparation is shown in Fig. 2b. The $S^{\circ}_{20,w}$ of this material was 11.1 S, the electrophoretic mobility was similar to that of γ-globulin.

All of 15 different preparations of the 11 S component proved highly active in precipitin tests with soluble γ-globulin aggregates. The activity of the isolated 11 S component was, like that of whole serum, thermolabile and independent of bivalent cations. To investigate whether the 11 S component was essential for complement activity of human serum, a serum rendered deficient in this component (R_{11S}) was analyzed for hemolytic activity. The R_{11S} was virtually devoid of hemolytic activity while comparable amounts of the original serum afforded lysis up to nearly 100% (Fig. 3). When a preparation of 11 S was added to an R_{11S}, its activity was fully restored. The required amount was less than 1 μg which corresponded to approximately 0.1% of total serum protein present. The activity associated with preparations of 11 S could not be identified with any of the classical components of complement as such preparations failed to reconstitute serum lacking either the first, second,

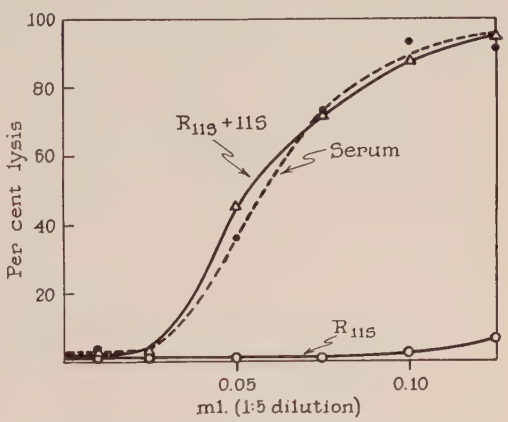

FIG. 3. Hemolysis curves demonstrating loss of hemolytic activity by depletion of a serum of 11 S component (R_{11S}) and regaining of full activity upon addition of a preparation of isolated 11 S.

third, or fourth component. Similarly, the lack of hemolytic activity of an R_{11S} could not be attributed to loss of any of the classical components, as none of them appeared to be significantly reduced. This included the first component, provided it was determined by an R_1 plus 11 S. In addition, preliminary evidence was obtained indicating that an intermediate complex, consisting of erythrocyte, antibody, and 11 S component can be formed and that this complex is capable of being lysed by an R_{11S} but not by other R's.

Discussion. Thermolability and independence of bivalent cations are intriguing features characterizing the activity associated with the 11 S component. The ability of this component to combine with γ-globulin aggregates in absence of bivalent cations suggests qualities more typical for an antibody than for complement. The similarity of its electrophoretic mobility with that of γ-globulin appears to lend further support to this view. However, the 11 S component is undoubtedly different from ordinary antibody protein. The sedimentation coefficient clearly distinguishes it from known types of antibody which have been shown to belong to the 7S and 19S class of serum proteins. Moreover, the 11 S component is markedly thermolabile while antibodies in general, with the possible exception of reagins, are resistant to heating at 56°C. Thus, it appears highly improbable that this protein represents an antibody, although this possibility cannot be ruled out at present.

The demonstrated participation of the 11 S component in immune hemolysis strongly suggests that it constitutes a component of complement. The fact that it is able to combine directly with γ-globulin, without the aid of another factor, and that it is absent from serum rendered deficient in the first component of complement(1,2), would relate the 11 S component to the first component. However, while fixation of hemolytic complement to immune aggregates requires Ca^{++} as has been shown by Levine *et al.* (9,10), combination of the 11 S component with γ-globulin aggregates does not. This, as well as the complete failure of preparations of 11 S component to restore hemolytic activity of an R_1 and the undiminished presence of C'_1 in an R_{11S} precluded identity with the first component.

The information at hand suggests that the interaction of the 11 S component with γ-globulin aggregates and with sensitized sheep cells precedes the action of the classical components. This concept is primarily supported by observations showing that interaction between this heat-labile component and soluble γ-globulin aggregates proceeds in the absence of Ca^{++}. It seems probable that, after the 11 S component has combined with aggregates, other factors of the complement system become able to interact with it, provided Ca^{++} and Mg^{++} are present. Certain combining sites of the 11 S component might thus be blocked by complement, preventing combination with other aggregates. In precipitation tests with aggregates, the amount of precipitate would therefore be expected to be considerably smaller in presence of Ca^{++} and Mg^{++} than in their absence. As pointed out above, this was actually observed. Further evidence will be needed to correct or to confirm the proposed concept according to which the 11 S component is part of human complement preceding in the reactions of complement the classical first component.

Summary. A protein of normal human serum capable of precipitating soluble aggregates of γ-globulin was isolated and found to be a euglobulin with an $S^{\circ}_{20,w} = 11$ S and the approximate electrophoretic mobility of γ-globulin. The activity of this protein proved thermolabile and independent of Ca^{++} and Mg^{++}. Serum rendered deficient in 11 S component showed drastically reduced hemolytic activity despite virtually unchanged titers for the classical components of complement, and regained full hemolytic activity upon addition of microgram amounts of preparations of isolated 11 S. The possible significance of this component for the early steps in complement reactions was discussed.

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Studies on the Metabolism of 5-Hydroxytryptamine (Serotonin). I. Effect of Starvation and Thiamine Deficiency. (26314)

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In spite of the significant number of important papers related to the metabolism of 5-hydroxytryptamine (5-HT) the effect of certain stresses notably those of starvation, dietary deficiencies and of obesity, are scarcely explored. An exception is the studies on the effect of Vit. B_6-deficiency which were shown to lead to reduced levels of 5-HT in the spleen(1), possibly through impairment of 5-hydroxytryptophan decarboxylase(2), and B_2-deficiency with concomitant reduction of monoamine oxidase (MAO) activity of the liver(3). The observed metabolic disturbances which appear during starvation(4) and thiamine deficiency (5,6) were considered to evoke possible changes in metabolism of 5-HT, a substance involved in functional control of the central nervous system. This paper derives from a research program concerning the influence of dietary stresses on certain aspects of 5-HT metabolism.

Methods and materials. Sprague-Dawley male rats were divided into 3 groups. The first 2 groups were kept on thiamine sufficient and deficient diet respectively for 4½ weeks as described elsewhere(7). The third group of rats was maintained on a daily ration of 2 g of Purina standard rat chow for the first 3 days, then starved for 4 days with only 1 ml of soluble vitamin mixture ("Berocca-C", Hoffmann-LaRoche, Inc.) and water given *ad libitum*. The animals were mechanically stunned and, after withdrawal of 1 ml of cardiac blood with a heparinized needle, killed by decapitation. Blood and tissues were immediately worked up for 5-HT content according to the method of Bogdanski *et al.*(8). In the experiments aimed at determining (MAO) activity the tissues were removed immediately and, with the excess blood blotted, chilled and worked up to isolate the mitochondrial fraction by the method of Schneider *et al.*(9). Mitochondrial fraction of the spleen was obtained by pooling samples from several animals. Measurement of MAO activity was carried out manometrically(10). The main compartment of the vessels contained 0.3 ml of 0.5 M phosphate buffer pH 7.3, 1 ml of mitochondrial suspension in 0.25 M sucrose, 0.1 ml of 3×10^{-2} M KCN solution and water to bring the volume to 3 ml, while the side arm had 0.4 ml of 0.05 M 5-HT solution adjusted to pH 7.4. The inner well contained filter paper, 0.2 ml of a 1:1 mixture of 20% KOH and 1 M KCN. Each experimental flask had a control in which the side arm had 0.4 ml of water instead of 5-HT. This served to correct for any oxidation in absence of substrate. The gas phase was O_2 and temperature 37°C. After 10 minutes of oxygenation and equilibration the contents of the side arms were tipped in. At the end of each experiment the content of main compartment was withdrawn for pH determination (average varied between pH 7.5 to 7.8). Enzyme activity is expressed as $Qo_2(N)$ and was calculated from the linear period of oxidation. Mitochondrial nitrogen was determined by micro-Kjeldahl method.

Results. Effect of starvation and thiamine

TABLE I. Effect of Starvation and Thiamine Deficiency on 5-HT Levels.

Tissue	5-HT levels, μg/g or ml Starved	Thiamine-sufficient (control)	Thiamine-deficient
Brain	.47 ± .03* (8)	.45 ± .04 (8)	.50 ± .04 (12)
Spleen	4.60 ± .53 (8)	1.75 ± .19 (8)	3.70 ± .38 (12)
		p < 0.01	p < 0.01
Small intestine	4.38 ± .50 (8)	4.18 ± .28 (8)	2.91 ± .23 (12)
			p < 0.01
Blood	1.02 ± .19 (10)	1.04 ± .11 (11)	.73 ± .06 (13)
			p = 0.01
Initial body wt (g)	143 ± 7	140 ± 6	141 ± 7
Final (% change)	−27	+33	−16

* Stand. error. No. of animals is given in parentheses.

deficiency on in vivo *levels of 5-HT*. Concentration of 5-HT recovered in the blood, brain, small intestine and spleen of rats under the specified dietary conditions is represented in Table I. It is of interest that neither starvation nor thiamine deficiency affected levels of 5-HT of the brain while there was a statistically significant increase of 5-HT concentration in the spleen of stressed animals. Among the organs analyzed, only the weight of the spleen of the deficient and starved animals displayed marked difference from that of control animals. Although weight of the spleen of these animals was 40% less per hundred g of body weight than the splenic weight of controls, the absolute amount of 5-HT was nevertheless twice as much in the spleen of the stressed animals. Degree of decrease in 5-HT concentration of the blood and small intestine was significant ($p < .01$) only in thiamine deficient animals. It should be pointed out that 5-HT values of rat blood indicated here are 3 to 4 times of those previously reported by others(8). Recent analyses of others have confirmed the blood values reported in these studies (private communication by H. Weisbach).

Effect of starvation and thiamine deficiency on 5-HT oxidase activity of rat tissues. The oxidation 5-HT by the mitochondria of liver, kidney and brain of normal rats as measured by the colorimetric method(11) was earlier demonstrated by Sjoerdsma *et al.*(12). Results of the manometric assays reported here seem to be in good agreement with respect to relative rate of oxidation of 5-HT found in those tissues of normal rats by the colorimetric method. There is significant difference in MAO activity of the brain and small intestine between control and the starved as well as the thiamine deficient animals (Table II). In the other tissues studied, notably liver, kidneys and spleen, no difference in MAO activity between control and experimental groups was detectable. In spite of the more than 40% loss of liver protein during starvation(13) the mitochondrial enzyme protein responsible for MAO activity does not seem to be impaired either in starvation or in thiamine deficiency. Earlier Sourkes (14) using 5-HT as substrate demonstrated the MAO activity of the liver of B_6-deficient rats was normal or occasionally even elevated. The results were qualitatively similar with tyramine as substrate. The comparison of the MAO values based on mg of mitochondrial nitrogen rather than wet weight of tissue increases their validity as a true measure of difference between the 3 groups.

TABLE II. Mitochondrial Monoamine-Oxidase Activity of Rat Tissues with 5-HT as Substrate.

Tissue	Q_{O_2} (N)* Starved	Thiamine-sufficient (control)	Thiamine-deficient
Brain	45 ± 3.7† (9)	31 ± 3.1 (9)	46 ± 3.8 (9)
		p < 0.01	p < 0.01
Small intestine	77 ± 11 (7)	28 ± 2.3 (8)	91 ±14 (8)
		p < 0.01	p < 0.01
Kidney	37 ± 11 (6)	36 ± 2.9 (9)	29 ± 5.6 (9)
Liver	70 ± 7.1 (8)	54 ± 7.8 (9)	55 ± 3.7 (9)
Spleen‡	26 ± 12 (4)	23 ± 5.6 (4)	32 ± 6.5 (4)

* Expressed as μl of O_2 consumed/mg mitochondrial nitrogen/hr.

† Stand. error.

‡ Each assay represents pooled tissues of 2 animals. No. of assays is given in parentheses.

Discussion. Acute starvation and thiamine deficiency, while non-specific stresses, provoke in the adrenals and thymus a definite response which is quite different from the effects of riboflavin and B_6-deficiencies. These latter fail to produce adaptation syndrome although in B_6-deficiency the atrophy of thymus is observable as a possible consequence of inadequate protein synthesis(5). With this in mind it was expected that the effects of starvation and thiamine deficiency through adrenal involvement would be reflected in changes in the metabolism of 5-HT. Furthermore, thiamine deficiency causes anemia by affecting the haemopoietic system (15) while starvation in rats produces a concentrating effect through body weight loss and dehydration(16) without apparent effect on haemopoesis. Of course such effect would also be operative in the ensuing inanition during the final stages of thiamine deficiency. The increased 5-HT levels of the spleen in the starved and deficient animals is therefore suggestive of platelet destruction and consequent release of serotonin as a result of inanition and concentrating effect of the spleen. However, the lowered 5-HT values of the blood and small intestine in the thiamine deficient animals might be related both to an increased release of 5-HT from the intestine to other organs only during chronic inanition like B_1-deficiency and to an increased MAO activity of the small intestine as shown in Table II.

The reason for the increased MAO activity of the brain and small intestine is difficult to interpret, particularly with respect to the starved animals. The loss of considerable protein from the intestines during starvation (13) and the significant decrease in number of cells in the duodenal mucosa(17) would indicate an imbalance in favor of protein catabolism which should tend to decrease enzymatic activity. On the contrary, it is obvious from the results that the enzyme protein of MAO was not adversely affected by either acute starvation or a 4 week period of thiamine deficiency in any of the tissues studied.

Summary. Blood and small intestine of thiamine deficient rats showed reduced levels of 5-HT while 5-HT content of the spleen of both starved and deficient animals was elevated. Mitochondrial monoamine oxidase activity was increased in the brain and small intestine during starvation and B_1-deficiency without any detectable change in that of the liver, spleen and kidney.

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Gonadotropin Secretion in Response to External Stimuli of Varying Duration in the Ring Dove (*Streptopelia risoria*).* (26315)

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Gonadotropin secretion in female ring doves may be induced by visual stimuli arising from the presence of male doves, and augmented by stimuli arising from the presence of a nest-site and nesting material(1,2). Female doves placed in cages with males show, over an 8-day period, increasing oviduct weight, increasing incidence of ovulation, and increasing incidence of behavior attributable to ovarian hormones, as compared with isolated controls; all these effects are increased by placing nest-bowls and nesting materials in the cages(2,3).

In all the above experiments, the males (and nesting material) have been continuously present in the cage during the period from the beginning of stimulation to occurrence of ovulation. The purpose of the present experiment is to determine whether the presence of these external stimuli is continuously necessary during the period of ovarian activity, or whether, on the other hand, they may start a process of ovarian development which, once started, can continue without the presence of the initiating stimulus.

Methods. 1. *Subjects.* The subjects were 260 female ring doves, all with previous breeding experience. Each of the birds had successfully reared young to the age of 21 days, after which the adults were placed in individual isolation cages for a period of 3-5 weeks. 2. *Experimental procedure.* All animal rooms were kept on a daily light cycle of 14 hours light (6:00 a.m.-8:00 p.m.) and 10 hours dark. All subjects were taken from the isolation cages at approximately 9:00 a.m. at start of experiment (day 0), and placed in test cages of the same type as the breeding cages. These were wood-and-wire cages 33″ wide, 18″ deep, and 14″ high, furnished with food, water and grit *ad lib.* All subjects were left in these cages for 7 days, then killed for autopsy. Twenty subjects were kept alone in the cages for the whole 7-day period. Each of the remaining 240 birds had a male in the cage with her during the first part of the 7-day period, after which the male was removed and the female left alone in the cage for the rest of the period. In 40 cases, the male was removed after 1 day, in 40 cases after 2 days, etc., up to 6 days. In each of these groups of 40 birds, 20 birds were initially supplied with a nest-bowl and a supply of nesting material, which were withdrawn at the same time as the male. 3. *Collection of data.* The data consist of observations of ovarian condition at autopsy after 7 days in the test cage. The 7-day interval between beginning of stimulation and time of autopsy was selected because previous work(4) has shown that over 90% of birds kept continuously with a mate and nesting material may be expected to have ovulated by that time.

Results. The results are presented in Fig. 1. It is apparent that incidence of ovulation

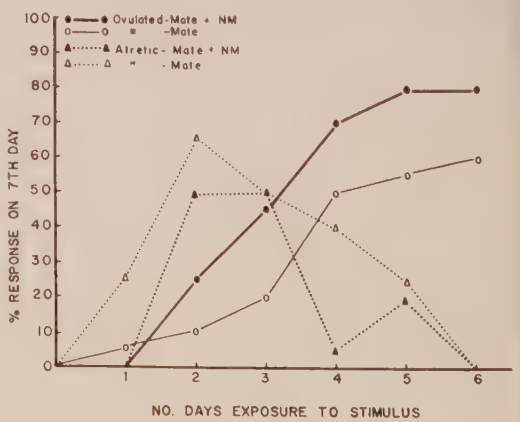

FIG. 1. No. of birds ovulating (solid lines) and No. of birds developing atretic follicles (dotted lines) by the 7th day, as a function of duration of exposure to a male (light lines) or to a male and nesting material (heavy lines) during the first 0-6 days.

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by the end of the 7-day period is a gradually increasing function of duration of exposure, at the beginning of the period, to the stimuli which induce secretion of ovulation-inducing hormones. This function is of approximately the same shape for birds stimulated by the male alone as for birds stimulated by the presence of a male and nesting material, except that the effect is augmented by the presence of nesting material from the 2nd day on.

Atretic follicles were noted in many of the ovaries, and incidences of these degenerating ova are plotted, for the various groups, as the dotted lines in Fig. 1.‡ For both types of treatment, the incidence of atretic follicles first rose, then fell, as a function of duration of exposure to the stimulating conditions. It should be noted that the incidence of atretic follicles is *lower* in birds supplied with nesting material than in those having only a mate.

In general, each bird could be characterized as having atretic or ovulated follicles, but not both: there were only 10 birds which had both atretic and ovulated follicles, compared with 66 birds with ovulated but not atretic, and 92 birds with atretic but not ovulated, follicles.

Discussion. These data confirm that stimulation provided by the mate, and by the presence of a nesting situation, induce gonadotropin secretion in female ring doves. When the mate and/or the nesting situation are continuously present, the effects of this stimulation culminate in ovulation, which occurs in almost all birds by the end of 7 days. This indicates, of course, the successive secretion of FSH and of LH by the bird's hypophysis(5).

The presence of atretic follicles under these circumstances probably indicates that the follicles started to develop under the influence of FSH, and degenerated when FSH secretion ceased. The increase in number of atretic follicles with increasing exposure to the stimuli up to 2-3 days of stimulation reflects the fact that increasing duration of stimulation induces development of increasing numbers of follicles which can become atretic when the stimulation is withdrawn. The decrease in incidence of atretic follicles with increasing duration of stimulation, after that point, occurs because increasing duration of stimulation causes increasing numbers of follicles to go on to ovulation even after the external stimulus is withdrawn.§ Somewhat similar considerations apply to the differences between the groups with, and those without, nesting material. The higher incidences of ovulations in the groups provided with nesting material indicate that the presence of nesting material augments the gonadotropin-stimulating effect of the mate; the *lower* incidence of atretic follicles in these groups results from the fact that this augmenting effect causes more of the follicles to go on to ovulation, even after the external stimulation is withdrawn.

We must note that birds of the age and previous experience used in this experiment are already secreting some gonadotropic hormone at the beginning of their exposure to the male; mere exposure to the 14-hour daily photoperiod induces ovarian growth which is augmented by the type of stimuli analyzed in this paper(6,7,8), although female doves never lay eggs when isolated from other birds.

It may be concluded that stimuli provided by the male and by the presence of nesting

‡ Ova in a mature ring dove ovary range in size from something less than 1 mm in diameter to the ovulation size of 15 mm. As in other avian ovaries the ova are clearly visible to the naked eye, suggesting a bunch of (small) white and (large) yellow grapes(10). In contrast to the spherical, bright yellow appearance of normal mature follicles, atretic follicles appear lobate or wrinkled, and vary in color from dark yellow to various shades of reddish and purplish(11,12).

§ That is, the increasing numbers of follicles which go on to ovulation leave decreasing numbers of unovulated mature follicles which *can* become atretic. An additional contribution to the decline in number of atretic follicles on the later days may be that the autopsies occurred too soon after withdrawal of the stimulus for atresia to become apparent. This factor must be relatively small, however, since 75% of the birds kept for 6 days with the mate and nesting material ovulated *both* eggs, and therefore would not have shown atretic follicles even if allowed more time before autopsy.

material stimulate follicular growth, but that this growth, and the progress of follicular development to its culmination in ovulation, can, once started, proceed without the presence of the original external stimuli. It is clear from the work of Fraps(9) that the developing follicle secretes hormones capable of exciting further activity by the bird's hypophysis. Obviously, the initial effect of the presence of the male is to stimulate FSH secretion. Any conclusions about the exact further effects of his presence, and of the presence of nesting material, must await further analysis.

Summary and conclusion. Female ring doves were exposed to stimulation by the presence of males and of nesting material for varying periods of time, from 0 to 6 days, after which these stimuli were withdrawn. The birds' ovaries were examined 7 days after the beginning of the period of stimulation. Consideration of the incidence of atretic follicles and of completed ovulations leads to the conclusion that the presence of the mate and of nesting material stimulates growth of ova, and that this growth, and the progress of the ova toward ovulation, gradually become independent of the external conditions which originally stimulated it.

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Tissue Oxygen Consumption in Rats Treated with Cortisone and with an Anabolic Androgen.* (26316)

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Hormones probably affect the cellular metabolism by modifying the activity of enzymes and by altering the permeability of the target cells(1). One of the characteristics of tissue metabolism related to enzyme activity is tissue respiration. Tissue respiration is, for example, decreased when the enzymes involved in catabolism of glucose are damaged(2). Tissue respiration may be influenced, both *in vitro* and *in vivo* by various hormones. Cortisone, a typical anti-anabolic (or catabolic) steroid, was found to alter Qo_2 in rats(3,4) and so did some anabolic androgens(5). It is apparent that certain anti-anabolic actions of cortisone on the metabolism of connective tissue may be offset by some anabolic steroids(6,7,8). It is known that the effects of some anabolic androgens on protein(9), carbohydrate(10) and mineral metabolism(11) and especially their promoting influence on growth and healing(6,7,8) are quite opposite to the metabolic action(12) of cortisone-like substances.

The purpose of this study was to find if the known metabolic antagonism between cortisone and an anabolic androgen occurs also in the action of these hormones on tissue respiration. We may expect that the in-

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TABLE I. Oxygen Consumption (Qo_2) of Diaphragm, Liver, Heart and Kidney of Rats Treated with Cortisone and 17-Ethyl-19-nortestosterone (ENT). Mean ± S.E.

Group	Treatment	Organ	No. of rats	No. of slices	Qo_2, $mm^3/mg/hr$
1	None	Diaphr.	72	162	6.00 ± .05
		Liver		193	7.14 ± .06
		Heart		203	8.54 ± .08
		Kidney		87	18.26 ± .15
2	Cortisone, 5 mg/day for 14 days	Diaphr.	18	58	* 4.73 ± .07
		Liver		64	* 6.07 ± .11
		Heart		46	* 5.76 ± .15
		Kidney		32	*16.35 ± .27
3	Cortisone, 10 mg/day for 14 days	Diaphr.	18	71	* 4.11 ± .06
		Liver		82	* 5.90 ± .07
		Heart		48	* 5.75 ± .09
		Kidney		58	*15.39 ± .14
4	*Idem*, then no treatment for 14 days	Diaphr.	12	39	5.94 ± .09
		Liver		50	6.65 ± .13
		Heart		37	8.60 ± .16
		Kidney		37	17.58 ± .26
5	ENT, 10 mg/day for 14 days	Diaphr.	16	60	* 4.78 ± .07
		Liver		60	* 5.91 ± .09
		Heart		53	* 5.70 ± .13
		Kidney		65	*14.78 ± .19
6	*Idem*, then no treatment for 14 days	Diaphr.	9	23	5.65 ± .08
		Liver		35	7.47 ± .12
		Heart		36	9.44 ± .29
		Kidney		30	18.95 ± .34

* P less than 1%: Tissues of treated rats *vs* comparable tissues of controls.

fluence of cortisone on Qo_2 may be related to the anti-anabolic action of this steroid on tissue metabolism. If so, what will be the response of tissue Qo_2 to a potent anabolic steroid, which is known to antagonize cortisone? This problem has been investigated here by comparing Qo_2 of some tissues in rats treated with cortisone and with 17-ethyl-19-nortestosterone.

Methods. Male Sprague-Dawley rats, weighing 225-272 g, kept in individual cages and fed special grain diet(13), were used for this study. Cortisone Acetate (Merck) was injected intramuscularly. Androgen, 17-ethyl-19-nortestosterone (ENT), (Searle & Co.) was given *per os,* in the form of crushed tablet mixed with food(7). Six groups of rats were treated as follows: 1—Normal controls, no treatment. 2—Given cortisone, 5 mg daily/rat for 14 days. 3—Given cortisone, 10 mg daily/rat for 14 days. 4—Given cortisone, 10 mg daily/rat for 14 days and then the treatment discontinued for another 14 days. 5—Given ENT, 10 mg daily/rat for 14 days. 6—Given ENT, 10 mg daily/rat for 14 days and then the treatment discontinued for another 14 days.

Rats were killed at end of experiment(5). Diaphragm, liver, heart and kidney were immediately excised and placed in cold nutrient medium, identical with the one used for Qo_2 study. Tissue slices were then prepared(5) and placed separately in reaction vessels containing 3 ml of oxygenated medium. Oxygen consumption (Qo_2) was measured by the direct Warburg technic(14) using the Krebs-Ringer phosphate buffer with glucose in an atmosphere of 100% O_2 at 37.0°C. Qo_2 was expressed in microliters of O_2 consumed per mg of dry tissue, per hour.

Results. The results of determination of Qo_2 are summarized in Table I. Treatment of rats with cortisone or with anabolic steroid 17-ethyl-19-nortestosterone significantly depressed the oxygen uptake of tissue slices studied. When, after 14 days of administration of any of these hormones, treatment was discontinued and animals left to recover for 14 days, (Groups 4 and 6) normal values of Qo_2 were found. Depression of Qo_2 found

in this study is therefore considered reversible.

Discussion. Expected antagonism between anti-anabolic (or catabolic) cortisone and anabolic androgen ENT was not found when the effect of these hormones on Qo_2 of various tissue slices was studied. Both cortisone and ENT depressed Qo_2 in diaphragm, liver, myocardium and kidney slices. These results agree with previous work on post-cortisone Qo_2 in the liver and heart(4) and on Qo_2 in the liver and diaphragm of rats treated with ENT(5). We were, however, unable to confirm the finding of Lacroix(3) who observed increased Qo_2 in the diaphragm of male rats treated with cortisone.

Cortisone and ENT are certainly antagonistic under various clinical and experimental conditions. It results from this study, however, that both these hormones had comparable action on tissue respiration. The depression of Qo_2 in tissues studied is reversible. Interruption of hormonal treatment for 14 days resulted in normalization of Qo_2.

More recent studies have shown that cortisone influenced glucose and pyruvate utilization and that the enzymatic impairment observed in tissues of cortisone treated rats is probably located at the level of the Krebs cycle. Cortisone also produced significant changes in enzymes involved in protein metabolism(4) and inhibited oxidative phosphorylation in liver mitochondria of rats (15). It may be expected that further studies of various enzymes, involved in cellular metabolism, will permit to find a difference between cortisone and anabolic hormones. Such studies are now in progress in this laboratory.

Summary. Treatment of male rats with cortisone or with anabolic steroid 17-ethyl-19-nortestosterone significantly depressed oxygen uptake of diaphragm, liver, myocardium and kidney slices. This depression of Qo_2 in tissues studied was reversible. Interruption of hormonal treatment for 14 days resulted in normalization of Qo_2. Expected antagonism between anti-anabolic and anabolic hormones was not found as far as the effect of these hormones on Qo_2 of tissues is concerned.

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Fluorescent Antibody Reactions Against the Mouse Mammary Tumor Agent.* (26317)

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Investigators(1-4) have shown that it is very difficult to prepare antisera against the mouse mammary tumor agent (MTA) and determine its reactivity by routine methods of titration. The method of titration normally used consists of a bio-assay technic in which antitumor sera is mixed and incubated with tissue extracts containing the MTA and given as a challenge to susceptible animals. A period ranging from 6 months to 2 years is necessary to determine the efficiency of the antisera to neutralize the agent. This preliminary investigation reports our findings with a fluorescent antibody system(5,7,8,9) which shows promise of drastically cutting to a few days the time period necessary for obtaining the results of the assay.

Technic. We have prepared antibodies against the Z(C_3H) tumor(6), which contains the agent, by injecting cellular suspensions of the tumor into animals of different species. A minimum of 5 rabbits and 12 guinea pigs being utilized for each serum preparation, wherein the animals received 6 doses of 3×10^8 cells/ml every third day, rested 10 to 12 days and exsanguinated. Pooled rabbit or guinea pig sera was used throughout the investigation. In these experiments the antigen consisted of Z tumor carried in Z strain mice, with normal Z mouse tissue serving as controls. Antisera to normal mouse tissue were prepared and tested in a similar fashion and this material, with normal rabbit guinea pig sera, served as further controls.

Thirty ml of anti-Z tumor sera were adsorbed with liver powder(7) prepared from non-tumored C strain mice which do not possess the MTA; This material with 30 ml of unadsorbed anti-Z tumor sera, was used for routine serologic, inhibition, and fluorescent-dye conjugation experiments. These two (adsorbed, fractionated, and whole sera) were both prepared from the same pools of anti-Z tumor sera. For agglutination tests a cellular suspension of the tumor of approximately 1×10^5 cells per ml was prepared from fresh tisue, while a cell-free homogenate of approximately the same number of original cells per ml was used for precipitin and complement-fixation tests.

Various fluorescent dyes were synthesized in the laboratory prior to use in these studies. Isothiocyanate was prepared by the method of Riggs(8) and Lissamine Rhodamine RB-200 isothiocyanate by the method of Chadwick *et al.*(9), as modified by Smith and associates(10). In addition, isothiocyanate and Lissamine Rhodamine RB-200 isothiocyanate samples were generously supplied by Major Chauncey W. Smith. No significant difference could be determined between the dyes made in this laboratory and those supplied by Major Smith. The method of conjugation was either that of Smith *et al.*(10, 11) when non-adsorbed whole sera were used, or that of Cherry, *et al.*(13) when adsorbed, ammonium sulphate fractionated sera were prepared.

Inhibition tests to determine ability of the antisera to inhibit tumor growth, prior to conjugation of the sera with fluorescent dyes, were set up according to the method of McCredie, Brown, and Cole(12). The technic was modified to the extent that 30 ml of the antisera (either guinea pig or rabbit) were mixed with freshly prepared tumor cells and incubated for one hour at 37°C. One ml of the preparation was inoculated s.c. into 15 Z mice. Normal rabbit sera and normal guinea pig sera were respectively incubated with Z tumor cells in a similar fashion as a

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† Post-doctoral Fellow of Am. Cancer Soc., Inc.

TABLE I. Inhibition Tests on Z Tumor Transplants in Z Mice.*

Material inj./mouse	Tested	No. of animals Developing tumor	No. of animals Not showing tumor
1 ml of 1 × 10^8 tumor cells + no antisera aged 30 min. at 37°C (Control)	15	15	0
1 ml of 1 × 10^8 tumor cells + normal guinea pig sera incubated 30 min. at 37°C (Control)	15	14	1
1 ml of 1 × 10^8 tumor cells + adsorbed anti-Z sera incubated 30 min. at 37°C	15	0	15
1 ml of 1 × 10^8 tumor cells + anti-Z g.p. sera (unadsorbed) incubated 30 min. at 37°C	15	3	12
1 ml of 1 × 10^8 tumor cells + adsorbed, fractionated anti-Z rabbit sera incubated 30 min. at 37°C	10	0	15
1 ml of 1 × 10^8 tumor cells + normal rabbit sera (fractionated and adsorbed) incubated 30 min. at 37°C (Control)	9	8	1

* All mice were of same inbred strain and approximate age. All animals were saved for 2 mo. Avg control animal developed tumors within 17 days. Equal volumes of antisera and cells were used for neutralizations.

control. In addition 15 Z mice were inoculated with the same preparation of tumor cells without prior serum treatment, but with the same degree of aging, *i.e.* 30 minutes after preparation of the cellular suspension. In each test or control series 15 mice/test were used, all from the same inbred stock and approximately the same age. All animals were held for a period of 2 months before concluding this aspect of the investigation.

For histologic work, slides were prepared from 10% formalin-fixed Z tumor by the method of Cherry *et al.*(13). The best results with the fluorescent stains were obtained when the tissue slices were 3 μ or less in thickness. At this depth maximum differentiation of tissue could be made. For each slide prepared for fluorescent staining, a slide from the next cut on the microtome was prepared for routine H & E histologic observation.

Results. The results of *in vitro* agglutination, precipitation and complement-fixation tests were inconclusive and no determination of titer could be made by these methods. In the case of agglutination tests, results with the antitumor sera ranged from negative to a titer of 1:5120 depending on the aliquot of the same antigen and sera tested. The observations with the other two technics were similarly inconclusive and it was difficult to determine when examining the test tubes whether or not antibody was present. Controls in which normal mouse tissue cells were used gave similar results despite the fact that some of the sera tested was adsorbed with normal mouse tissue preparations to remove common antibodies. When the antigens used in these tests were reacted against normal rabbit or guinea pig sera, results were again inconclusive.

Results of the inhibition tests, were, however, more promising (Table I). It should be noted that these tests utilize viable tumor cells for transplantation of the tumor whereby the tumor usually develops within 2 to 3 weeks in the new host. In bio-assaying the mouse mammary tumor agent(6) a suspension of normal or cancerous tissue containing the agent is used for the challenge. In this case, a minimum of 6 months to 2 years is necessary to determine whether or not the agent will have been neutralized by the antisera.

When conjugated anti-Z rabbit or guinea pig sera were used for *in vitro* testing the results were more pronounced. The use of Lissamine Rhodamine RB 200 conjugated to normal albumin and mixed with the antisera conjugated to isothiocyanate essentially reduced the nonspecific fluorescence of the tissue substance. Use of carefully adsorbed and fractionated (ammonium sulfate) anti-Z sera gave reproducable results when tested against various slides of Z tumor. In general, specific staining by fractionated and adsorbed anti-Z

sera coupled with Lissamine Rhodamine RB 200 isothiocyanate was enhanced due to the contrasting reddish-orange background. When the conjugated antisera were adsorbed and concentrated by the method of Cherry *et al.* (13) and then reacted with normal mouse tissue no specific fluorescence could be distinguished. This was true with both the Lissamine Rhodamine RB 200 isothiocyanate and the isothiocyanate conjugated normal rabbit or guinea pig sera. When the conjugated anti-Z tumor sera were tested against a mouse sarcoma and an adenocarcinoma of different origin from the Z tumor (*i.e.*, did not involve the MTA and different mouse strains were involved) no specific fluorescence was noted. Such cancers, similar to normal tissue controls in regard to degree of fluorescence, were always observed whenever Z tumor was tested. A direct correlation was found between use of fluorescein-labeled antisera and the inhibition tests described. When the conjugated antisera were reacted with normal tissues no specific fluorescence was observed. However, it was found that when unadsorbed anti-Z sera was used that antibodies common to normal tissue as well as to the Z tumor were present, making the slides difficult to interpret. This difficulty could be overcome by judicious adsorption with normal mouse liver powder.

Discussion. Preliminary tests show that the fluorescent antibody technic can be adapted to study of the mouse mammary tumor and the mouse mammary tumor agent. This method offers a more reliable technic for *in vitro* testing of antisera specific against the MTA or mouse mammary tumor containing the agent than our present serologic tests. The best methods of fluorescent staining were found to be either use of isothiocyanate or the Lissamine Rhodamine isothiocyanate technic of Smith and his colleagues (10). Direct correlation was found between ability of the sera to inhibit transplant of Z tumor in Z mice and to show specific fluorescence when conjugated anti-Z sera was used against homologous tissue. The same sera which would inhibit tumor growth, *i.e.*, neutralize, would also show specific fluorescence when conjugated to the appropriate dye. Tests are now underway to determine the specificity of the fluorescent antibody technic as compared to bio-assay of nontumor material containing the MTA.

Interpretation of fluorescein-stained slides is not necessarily a simple technic. As Hughes(14) has mentioned in his work on differentiating neoplastic and non-neoplastic tissues, similar results can be obtained with fluorescein conjugated normal rabbit globulin as with fluorescein labeled protein complexes. Any interpretation of results obtained by the fluorescent technic requires caution and carefully controlled staining when evaluating changes attributed to an antigen-antibody complex. Smith *et al.*(8) point out, "as the LR-labeled normal sera react under these circumstances, it is presumed to be a protein-protein physicochemical interaction, depending on factors other than serological. This system now makes it possible not only to better visualize the sites of antigen deposits but also to detect smaller bacteria and viral aggregates in formalin-fixed tisues and tissue cultures." We have found this to be generally true but careful choices must be made in selecting adsorbing tissue, in preparation of the tumor antigen, and especially in interpretation of the slides.

Summary. A method of assaying the potency of sera prepared against the mouse mammary tumor agent using fluorescent dye labeled antisera is described. Better correlation between this method and serum neutralization tests, with transplanted Z tumor in Z strain mice, were found than in routine serologic testing. Agglutination, precipitin, and complement-fixation tests were found to be completely unreliable when testing anti-Z tumor sera. Use of Lissamine-Rhodamine RB-200 and isothiocyanate as described by Smith *et al.*(8) eliminated most nonspecific fluorescence and gave a better analysis of the reactive sites. Similar results were found using antisera which had been adsorbed with normal tissue and the globulins fractionated by ammonium sulfate and then conjugated with isothiocyanate. Little or no reaction was observed when either of these materials was tested against normal tissues or tumors

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Deleterious Effects of High Fat Diets on Survival Time of X-Irradiated Mice.* (26318)

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Considerable data are available indicating that sensitivity to total body x-irradiation is enhanced in animals fed diets deficient in fat. Cheng *et al.*(1,2) reported that the average survival time of rats fed a fat-free diet was significantly less following multiple sublethal doses of total body x-irradiation than that of animals fed a similar diet supplemented with cottonseed oil or methyl linoleate. Similar findings have been reported for the mouse(3). It has also been observed that whereas a diet containing 10% cottonseed oil resulted in longer survival following multiple sublethal doses of total body x-irradiation than occurred in mice fed a diet deficient in fat, higher levels of fat (*i.e.*, 20% or 30% cottonseed oil in the ration) decreased survival time to that of mice on the fat-free diet.† These studies were confirmed and extended in the present investigation.

Procedure. The basal fat-free ration employed in these studies consisted of cerelose, 69%; casein,‡ 24%; salt mixture,§ 5%; cellulose,‖ 2%; and the following vitamins per kilogram of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; Vit. B_{12}, 150 μg; 2-methyl-1, 4-naphthoquinone, 5 mg; choline chloride, 2 g; Vit. A, 5000 U.S.P. units; Vit. D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of an equal amount of cerelose. Tests were conducted with mice fed the basal fat-free ration or the basal fat-free ration supplemented with 2%, 10%, 20% or 30% of the following fats: cottonseed oil, margarine fat¶ or butter fat,¶

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† Ershoff, B. H., unpublished data.

‡ Vitamin-Free Test Casein, General Biochemicals, Inc., Chagrin Falls, O.

§ Wesson Modification of Osborne-Mendel Salt Mixture, General Biochemicals, Inc., Chagrin Falls, O.

‖ Solka Floc, Brown and Co., Boston, Mass.

¶Margarine fat and butter fat were prepared by the procedure of Deuel *et al*(5). Margarine fat was prepared from Nucoa (Best Foods, Inc., New York, N. Y.); butter fat from butter (Arden Farms Co., Los Angeles, Calif.).

TABLE I. Comparative Effects of Graded Levels of Cottonseed Oil, Margarine Fat and Butter Fat on Survival Time of Mice Exposed to Multiple Sublethal Doses of Total Body X-irradiation.

Dietary group	No. of animals	Day after 1st x-irrad. when 50% of animals in group were dead	% survival*	Avg survival time, days†‡
Basal fat-free diet	25	42	0	46.3 ± 3.0
Idem + following supplements:				
2% cottonseed oil	24	54	33.3 (8)	66.0 +
10% " "	24	>103	75.0 (18)	95.2 +
20% " "	26	41	19.2 (5)	55.5 +
30% " "	26	32	15.4 (4)	45.5 +
2% margarine fat	30	56	30.0 (9)	68.7 +
10% " "	28	93	46.4 (13)	76.2 +
20% " "	25	51	20.0 (5)	59.6 +
30% " "	27	41	22.2 (6)	53.1 +
2% butter fat	28	39	0	40.3 ± 1.1
10% " "	28	39	3.6 (1)	41.3 +
20% " "	28	46	3.6 (1)	50.9 +
30% " "	26	50	30.8 (8)	62.6 +

* Experiment was terminated 103 days after first x-ray exposure. Values in parentheses indicate No. of animals which survived.

† Data were calculated on basis of a 103-day survival time for animals alive at termination of experiment.

‡ Including stand. error of mean.

which were added in place of an equal amount of cerelose.

Male mice of the Webster strain, 11 to 14 g in body weight, were divided into comparable groups of 40 animals per group. These were placed in metal cages with raised screen bottoms (5 animals per cage) and were fed the test rations indicated above. Food and water were provided *ad libitum*. The animals were fed daily and all food not consumed 24 hours after feeding was discarded. After 6 weeks of feeding, 10 of the mice in each dietary group were selected at random to serve as non-irradiated controls. The remaining mice received an exposure of 200 r total body x-irradiation which was repeated once weekly until a total dose of 1200 r (6 exposures) had been administered. Animals were continued on their respective diets for 103 days after the first x-ray exposure or until death, whichever occurred sooner. Mice that died before the 4th x-ray exposure were not included in the tabulation of data. Animals were weighed weekly during the experiment and data were obtained as to the day after the first x-ray exposure when 50% of the animals in each group were dead, average length of survival and percentage of animals in each group still alive at termination of experiment.

The following radiation factors were employed: GE Model Maximar 250; 250 kv; 15 ma; 0.5 mm Cu and 1 mm Al filters plus a Cu parabolic filter;** HVL, 2.15 mm Cu; target distance to top of box, 82 cm; and dose rate, 15.6 r/min (measured in air, at top of box). The animals to be irradiated were placed in a wooden box divided into 60 equal compartments 1¼″ wide, 3″ long, and 1½″ deep. The partitions and top were made of ⅛″ cellulose acetate sheeting; and the top and bottom of each compartment were perforated with holes for purposes of ventilation. The container was rotated slowly on an electrically driven turntable to ensure equivalent irradiation.

Results. Findings indicate that average survival time of mice following exposure to multiple sublethal doses of total body x-irradiation is dependent on both the amount and source of dietary fat (Table I). In

** A nonuniform filter which produces a flat isodose surface of x-ray intensity constructed by the method of Greenfield and Hand(6). The center of the filter had a thickness of 1.7 mm Cu; the edge, 0.5 mm Cu.

agreement with earlier findings(3) a supplement of 2% cottonseed oil markedly increased average survival time of x-irradiated mice over that of animals fed the basal fat-free ration alone. At the 10% level of supplementation this increase was even more marked. Whereas 50% of the mice in the fat-free group were dead on the 42nd day after the first x-ray exposure, at the 2% level of cottonseed oil supplementation 50% mortality did not occur until the 54th day; while at the 10% level of cottonseed oil supplementation 75% of the animals in the group were still alive at termination of the experiment 103 days after the first x-irradiation. At higher levels of cottonseed oil supplementation, however, (*i.e.*, 20% or 30% of the diet) the protective effect of cottonseed oil on survival of x-irradiated mice was no longer evident, with average survival time on such diets not differing significantly from that of mice on the basal fat-free ration. A similar trend was observed in animals fed the various levels of margarine fat. In contrast to the results obtained with the cottonseed oil and margarine fat supplements, butter fat at levels of 2% or 10% of the diet did not increase survival following x-irradiation over that on the basal fat-free ration alone (a finding that may have been due to its low content of essential fatty acids). In addition butter fat at levels of 20% or 30% of the diet prolonged survival over that obtained at the 10% level of supplementation, a finding also in contrast to that obtained with the cottonseed oil and margarine fat supplements. All non-irradiated mice fed the cottonseed oil and margarine fat supplements survived the experimental period of 145 days (103 + 42 days). From 70 to 90% of the non-irradiated mice fed butter fat at levels of 10% of the diet or higher also survived. However, only 3 of the 10 non-irradiated mice fed butter fat at a 2% level in the diet and only 2 of the non-irradiated mice on the basal fat-free ration were alive at termination of the experiment, average survival time of the decedents being 126 and 123 days respectively (*i.e.*, equivalent to 84 and 81 days respectively after the first x-irradiation).

The cause for the deleterious effect of high levels of cottonseed oil and margarine fat on survival of x-irradiated mice is not readily apparent. It has been proposed that the deleterious effects of x-irradiation are due, at least in part, to excessive formation of free radicals(4). It is possible that the ingestion of unsaturated fatty acids in excess of body requirements for the essential fatty acids might result in the x-irradiated animal in the formation of more free radicals than might otherwise occur on diets containing less of these fats. If such were the case, survival time of x-irradiated mice on a fat-free diet supplemented with an unsaturated fat containing essential fatty acids might be expected to increase with increasing amounts of fat until a level is reached which provides an optimum amount of essential fatty acids; and thereafter decrease due to formation of excessive amounts of peroxides and free radicals. This suggestion might account for the effects obtained in the present experiment with cottonseed oil (which contains a high level of unsaturated fatty acids) and butter fat (which has a minimal amount of such acids); but the similarity of results obtained with cottonseed oil and margarine fat, despite their difference in unsaturated fatty acid content, is not consistent with this hypothesis. Since margarine fat is made from vegetable oils, it is possible that such oils contain a factor (or factors) other than unsaturated fatty acids which is toxic when fed at high levels to the x-irradiated mouse or results in formation of a toxic substance(s) or metabolite as a consequence of x-irradiation in the animal's tissues.

Summary. The effects of multiple sublethal doses of total body x-irradiation were determined on survival time of male mice fed either a purified fat-free ration or a similar diet supplemented with 2%, 10%, 20% or 30% cottonseed oil, margarine fat or butter fat. The effects obtained were dependent on the amount and source of dietary fat. At levels of 2% or 10% of the diet cottonseed oil and margarine fat increased survival time over that on the fat-free ration. When these fats were fed at higher levels (*i.e.*, 20% or 30% of the diet), however, survival time was

decreased below that obtained at the lower levels of supplementation. In contrast to the results obtained with the cottonseed oil or margarine fat supplements, butter fat at levels of 2% or 10% of the diet did not prolong survival over that on the fat-free ration; nor did it decrease survival time when fed at higher levels in the diet.

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Histochemical Localization of L-Gulonolactone Oxidase Activity in Tissues of Several Species.* (26319)

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Oxidation of L-gulonolactone by a specific oxidase is the final step in the pathway of synthesis of L-ascorbic acid from glucose(1). Coenzymes I and II do not participate in the reaction. The transfer of electrons is probably mediated by a flavoprotein the exact nature of which has not been defined. L-gulonolactone oxidase is confined to the liver of mammals, and to the kidneys of amphibia and reptiles(1). According to some investigators it is present only in microsomes(2) while others claim to find some activity in mitochondria(3). It is the absence of L-gulonolactone oxidase and consequent inability to synthesize Vit. C which results in the susceptibility of humans, monkeys, guinea pigs and a few other species to scurvy. In this paper we describe a histochemical method which demonstrates the distribution of the enzyme in tissue slices.

Materials and methods. Thirty adult white rats (250 g) and 10 adult guinea pigs (350 g) on standard laboratory diets were killed by a blow on the head and the tissues immediately removed, blocks 5 mm by 1 cm made, and frozen on dry ice. In addition kidney and liver tissue were obtained from 6 frogs (*Rana pipiens*) and treated similarly. Within 30 minutes of freezing the blocks were cut in a cryostat (–18°) at 20 to 34 μ thickness. Sections were put on coverslips and allowed to rinse for 5-10 minutes in cold (4°C) 0.03 M KCl solution. Then they were removed to an incubating mixture which consisted of 15 mg L-gulonolactone, 2.5 mg of the paranitrophenyl substituted ditetrazolium salt (Nitro-BT) in 4.75 cc of 0.1 M phosphate buffer pH 7.4. The solution was brought up to 5 cc by addition of 0.25 cc of acetone in which was dissolved 0.5 mg of menadione (Vit. K). The incubating mixture was warmed to 37°C before introduction of the sections. The tissues were then incubated at 37°C for 20 to 40 minutes. At termination of the incubation, the medium was drained off and replaced by 10% neutral formalin in which the sections were allowed to fix for 1 hour at room temperature. Sections were then placed on slides using glycerogel, and examined under the light microscope.

Control sections were incubated in solutions which individually excluded substrate and other components of the incubating mixture.

In some experiments sodium pyrophosphate or ethylene diamine tetra-acetic acid (EDTA) at varying concentrations were included in the incubating mixture.

Results. Rat liver was the only mammalian organ studied in which L-gulonolac-

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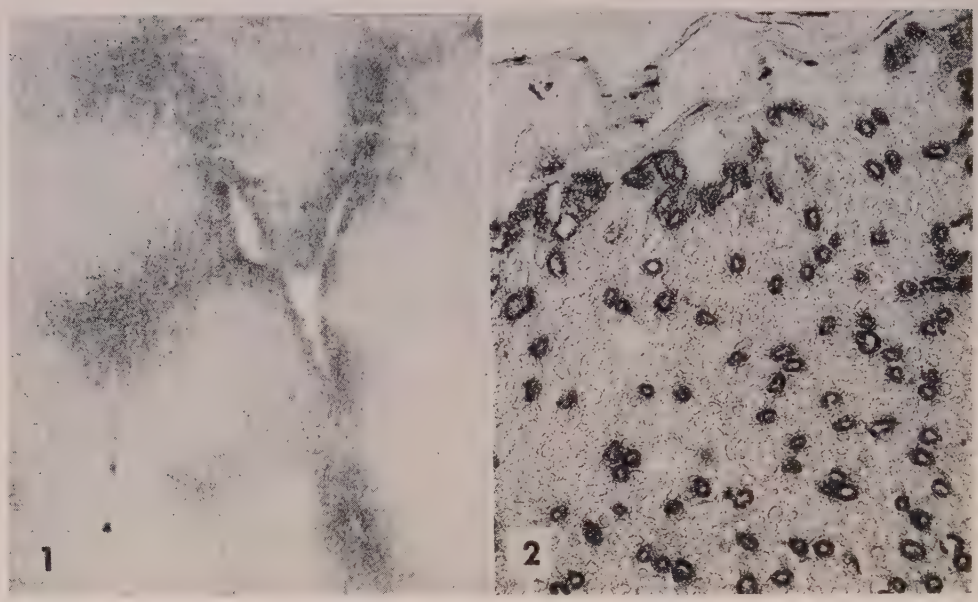

FIG. 1. A section of rat liver showing L-gulonolactone oxidase activity distributed predominately in cytoplasm of epithelial cells around the central vein. Bile ducts, connective tissue and blood vessels are all negative. ×40.

FIG. 2. A section of frog kidney showing selective distribution of L-gulonolactone oxidase activity in cytoplasm of epithelial cells of mesonephric tubules. ×50.

tone oxidase activity was demonstrated. This enzyme was confined largely to the cytoplasm of the centrilobular cells (Fig. 1). Bile ducts, blood vessels and connective tissue were negative as well as kidney, spleen, gastrointestinal tract, lung, ovary, adrenal, uterus and all nervous tissue.

As might be expected all guinea pig organs including liver and kidney were negative.

In the frog only the kidney showed L-gulonolactone oxidase activity. The distribution was confined to the cytoplasm of cells of the more distal portion of the mesonephric tubule, and enzyme activity was completely absent from a considerable portion of the tubule (Fig. 2).

The controls in which the incubating mixture lacked L-gulonolactone were uniformly negative, thus ruling out reduction of the tetrazolium salt *via* the oxidation of endogenous substrates or other endogenous reductants. Menadione definitely increased enzyme activity. EDTA or sodium pyrophosphate enhanced enzyme activity slightly in absence of menadione, but did not increase activity over and above the increase provided by menadione.

Discussion. The mechanisms involved in the histochemical demonstration of oxidative enzyme activity by use of tetrazolium salts as indicators have been discussed elsewhere(4). In the system described here the sequence of events includes oxidation of L-gulonolactone with transfer of electrons through one or more intermediates and ultimate reduction of the Nitro-BT to form a blue granular insoluble substantive diformazan at site of enzyme activity.

It has been demonstrated that the lactone of L-gulonic acid and not the acid itself is the required substrate for the oxidase. L-gulonic acid may be converted alternatively *via* another metabolic pathway to L-xyulose(1). An aldolactonase present in liver and other tissues(5) might conceivably have interfered with the oxidase reaction in our system by splitting the lactone to the acid which would then not have reacted. This, however, can be excluded as a serious limitation because of the apparent excess of substrate. In addition EDTA at concentrations of .001 M which inhibits aldolactonase activity(5) did not appreciably augment the L-gulonolactone oxidase activity as demonstrated by this histochemical technic.

The mechanism of action of menadione in

enhancing enzyme activity is not clear. It has been recently shown that it has a similar enhancing effect on the activity of several other oxidases in the liver(6).

The absence of L-gulonolactone oxidase activity in the liver of guinea pig and amphibians, and its presence in frog kidney as determined by other technics(1) offer an excellent biological control for the histochemical method by the use of which we found an identical general distribution and which provided the advantage of histological localization of activity.

Summary. A histochemical method was described which localized the activity of L-gulonolactone oxidase, the final oxidative step in synthesis of ascorbic acid from glucose. The enzyme was found to be active in the centrilobular cells of the rat liver and in selected portions of the mesonephric tubules of the frog kidney. It was absent from guinea pig kidney and liver, and from frog liver.

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Latent Viral Infection of Cells in Tissue Culture. IX. Abortive Infection with Psittacosis Virus.* (26320)

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A latent infection of L cells with psittacosis virus has been described which is induced by nutritional depletion of cells with a balanced salt solution (BSS) for 2 days prior to infection(1). Following entry into the cell the virus cannot be detected by infectivity tests in chick embryos, but addition of a synthetic medium containing amino acids and vitamins results in reappearance of infectious virus(2). The specific amino acid requirements for conversion of the latent infection to an active one have been delineated(2) and deletion of a single essential amino acid from the medium was shown to be sufficient to render L cells incapable of supporting psittacosis virus propagation and to establish a latent infection.

Since L cells survive longer on a medium deficient in a single amino acid than on BSS alone, studies were initiated to determine if the duration of the latent infection could be extended to permit a more detailed study of its nature.

Materials and methods. Psittacosis (6BC strain) virus stocks were prepared as previously described(3) and diluted in the appropriate amino acid-deficient culture medium for inoculation of the cell cultures. The single dilution method of Golub(4) was used for virus assay in which 0.25 ml of a 10^{-1} dilution of culture fluid was inoculated into the yolk sac of a dozen 7-day-old chick embryos. Virus titers were expressed as the $\log_{10}LD_{50}$ per ml. Cultures of L cells were grown in T-15 flasks in Parker's medium #199 supplemented with 10% horse serum. When a uniform sheet of cells had grown over the glass surface, the growth medium was replaced with a synthetic medium which was deficient in either phenylalanine or isoleucine. Cultures containing approximately 2×10^6 cells were exposed to approximately 10^5 LD_{50} of psittacosis virus after 2 or 3 days' mainte-

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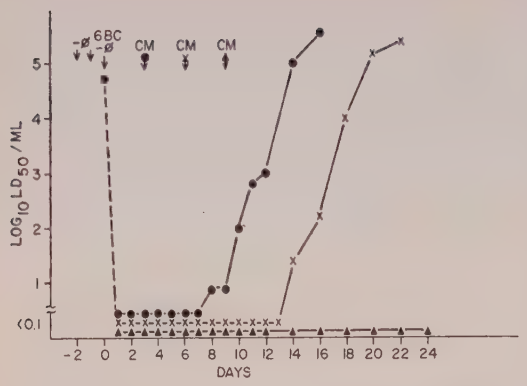

FIG. 1. L cells were maintained on phenylalanine-deficient medium ($-\phi$) for 2 days before infection with 6BC psittacosis virus. At intervals after infection, a complete medium was added and culture fluids were tested for recovery of virus.

nance on the deficient medium. At intervals after infection, the complete synthetic medium (CM), containing amino acids, vitamins, glutamine, glucose, and inorganic salts (2), was added. Culture fluids were tested daily for virus infectivity and the morphological condition of the cells was recorded. Cells were examined for content of virus following lysis by freezing and thawing and inoculating the lysate into eggs as in the technic used for titration.

Results. L cells which were maintained on a phenylalanine-deficient medium for 2 days prior to infection did not support psittacosis virus propagation. If such infected cells were lysed by freezing and thawing 24 hours after inoculation with virus, no virus was detectable by infectivity tests in chick embryos. The possibility that small amounts of virus might be present and would not be sufficient to kill the chick embryos on initial inoculation was eliminated by preparing yolk sac suspensions from test chick embryos which were injected into additional embryos which also survived. When the complete synthetic medium (CM) was added to the L cell cultures 3 days after infection, virus reappeared in the culture fluids within a few days and degenerative changes typical of virus action were eventually observed (Fig. 1). If a 6-day interval between infection and addition of complete medium was allowed, a longer time was required for virus to become detectable in the culture fluids. By the 9th day after infection, cells maintained on phenylalanine-deficient medium had become rounded and some loss of cytoplasm was noted. A few cells had disengaged from the glass surface, but this occurred also in noninfected controls. Although addition of the complete medium brought about a morphological recovery of the cells, virus was not released into the culture fluids and no cytopathic effects were noted in a 19-day observation period. When these recovered cultures were reinfected, the virus grew readily and produced complete disintegration of the cultures.

Although 3 days' maintenance on isoleucine-deficient medium was necessary before addition of virus to establish a latent infection, the resulting phenomena were similar to those described for phenylalanine depletion. Isoleucine-deficient cells retained their normal morphological characteristics longer than cells on phenylalanine-deficient medium, but the latent infection with the virus could not be extended beyond 8 days. After this time cultures fed with complete medium produced no virus, but on reinfection supported viral multiplication and were destroyed.

Discussion. Extension of the interval between infection and the addition of virus-stimulating culture medium to amino acid-depleted L cells *in vitro* did not result in extension of the period of latency beyond 6 to 8 days. The previous report(3) of a latent period lasting as long as 14 days after infection of depleted chick embryo tissue fragments is probably a reflection of a slower diffusion of nutrients from the cells in the fragments and possibly a relatively lower metabolic turnover, since a 13-day depletion period was required before the latent state could be established in such preparations.

It is apparent that the virus does not recover from its latent phase with extended metabolic depletion of L cells and therefore that the fate of the infecting particle depends upon the metabolic activity of the host cell. Amino acid deficiency in L cells prevents completion of the infectious cycle of psittacosis virus with establishment of a latent state for up to 8 days and eventual cure of infection, and survival of cells which retain

their susceptibility to reinfection. The possibility that all of the cells initially infected had lysed leaving only uninfected survivors is improbable, since toxic reactions of L cells to high multiplicities of psittacosis virus have not been noted in the absence of virus multiplication(5) and the slight decrease in infected cells during phenylalanine or isoleucine depletion was not observably different from that seen in noninfected cultures.

Summary. L cells which are maintained for 2 or 3 days on phenylalanine- or isoleucine-deficient medium do not support the growth of psittacosis virus and a latent infection is established. Virus can be recovered from these cultures only after addition of a complete medium, but extension beyond 8 days of the interval between infection and addition of complete medium results in a permanent disappearance of infectious virus. Recovered cultures are susceptible to reinfection with psittacosis virus.

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RBC Survival in Hamsters Using Intraperitoneal $Na_2Cr^{51}O_4$.* (26321)

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The use of radioactive chromium (Cr^{51}) for determination of red cell longevity has been well established both in man and animals. The technic usually employed for labelling erythrocytes involves incubation of blood *in vitro* with $Na_2Cr^{51}O_4$(1,2,3). The intravenous route of administration has commonly been used to return the labelled red cells to the circulation, in expectation of obtaining the most predictable and highest level of specific radioactivity(4,5,6).

Performance of erythrocyte survival studies in small animals is relatively more difficult than in larger animals or in humans because of limitations imposed by small circulating blood volume and the technical difficulty of returning labelled red cells intravenously. Accordingly, in our studies of the anemia of tumor bearing hamster, another method of erythrocyte chromation was tested, namely *in vivo* chromation with intraperitoneally administered $Na_2Cr^{51}O_4$. This method has proved eminently satisfactory and is the basis for this report.

Materials and methods. Golden hamsters (*Misocricetus auratus*) weighing 60-80 g were fed Purina Laboratory Chow and water *ad lib.* and kept in an air-conditioned animal room.

An aqueous solution of $Na_2Cr^{51}O_4$† with specific activity of 0.25 to 1.0 millicuries per mg of chromium metal was diluted with an appropriate volume of normal saline just before injection, so that an injectable dose of 30 μc of Cr^{51} was contained in approximately 0.3 ml of final solution. This dose of radioactive chromium was injected intraperitoneally into each of 7 hamsters. Twenty-four hours later and thereafter at 2 to 7 day intervals 0.1 ml of whole blood was obtained by cardiac puncture using a 22-gauge needle and a tuberculin syringe. This sample was placed in 0.5 ml of normal saline in a graduated centrifuge tube and the volume adjusted to 1.0 ml by addition of normal saline. The samples were then counted in a well-type

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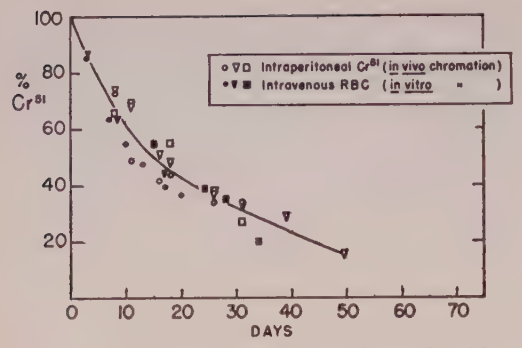

FIG. 1. Hamster red blood cell survival using 30 μc Cr^{51} ($Na_2Cr^{51}O_4$).

scintillation counter (sodium iodide thallium-activated crystal in an Atomic Ins. Co. scintillation head). Photoelectric hemoglobin determinations were performed on each counted sample using a Coleman 6 spectrophotometer. Chromium^{51} counts were computed in terms of specific hemoglobin activity *i.e.,* counts per minute per mg of hemoglobin. The erythrocyte survival curve in 3 hamsters was constructed by plotting % of Cr^{51} against time.

The erythrocytes were sedimented from the plasma in 4 hamsters and each of these portions was counted separately. Blood samples from animals injected intravenously with $Na_2Cr^{51}O_4$ were also divided into plasma and cellular portions before counting.

Results. Chromium^{51} counts varied from 250 to 12,000 per 0.1 ml whole blood per minute, with background counts of 130 to 150 per minute. Radioactivity was detectable in the circulating plasma as well as the red blood cells from 4 to 6 days following intraperitoneal injection, after which significant counts were present exclusively in the erythrocytes. A similar loss of plasma counts was seen following intravenous injection of sodium chromate^{51}. For this reason the fourth day was chosen as a reference point and the curves were extrapolated from this point to the time of intraperitoneal injection. The resulting mean erythrocyte survival curve in normal hamsters is seen in Fig. 1. The half period of red cell radioactivity ranged from 12 to 20 days and the extinction point, representing total red cell longevity, was extrapolated to be from 60 to 70 days. Uptake of Cr^{51} by circulating RBC following intraperitoneally injected $Na_2Cr^{51}O_4$ was in the range of 14 to 20%. Labelling of circulating red cells was completed within a period of 72 hours, as indicated by the fact that no subsequent variations in individual survival curves were encountered.

Discussion. The erythrocyte survival curve (Fig. 1) obtained after intraperitoneal injection of Cr^{51} is quite similar to that obtained by intravenous injection of red cells labelled *in vitro* by the technic of Ebaugh *et al.*(1). However, uptake of Cr^{51} by hamster erythrocytes *in vitro* was significantly less than that which is usually obtained under similar conditions with human erythrocytes, and relatively higher specific radioactivity in the erythrocyte mass could be obtained by chromation *in vivo.*

Preliminary studies in other animals suggest that this simplified technic of erythrocyte chromation *in vivo* may also be applicable to mice, rats, and other small laboratory animals. It has been successfully employed in the dog(7). Its usefulness in other large animals remains to be explored.

Conclusion. 1. Intraperitoneal administration of sodium chromate^{51} results in the effective chromation *in vivo* of red cells in the golden hamster. 2. With use of this labelling technic the erythrocyte survival of the normal golden hamster has been shown to be between 60 to 70 days. 3. The advantages of tagging erythrocytes *in vivo* include relative ease of administration of chromium and elimination of the necessity of obtaining a large initial blood sample.

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Effects of Renal Pressor System During Hemorrhagic Hypotension.* (26322)

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During hemorrhagic shock, normal animals have larger amounts of circulating renin(1), better maintenance of blood pressure and longer survival periods(2) than nephrectomized animals; these observations have been considered as evidence for the participation of the renal pressor system. However, development of tachyphylaxis and decrease in plasma renin-substrate, source of the pressor agent angiotensin, led others(3,4) to doubt the efficiency of this emergency mechanism. Furthermore, it should be realized that some of these observations were based on determinations of the various components of the renal pressor system in blood for which there is yet no reliable or accurate method. In the present paper we measured pressor effects of kidneys and demonstrated that in rats with hemorrhagic hypotension renal pressor substances play a definite role in homeostatic regulation of blood pressure.

Methods. Normal kidneys were grafted onto bilaterally nephrectomized rats during hemorrhagic hypotension. A method of grafting without interruption of the renal circulation has been described(5). Femoral blood vessels of a nephrectomized recipient rat are first anastomosed with lumbar aorta and vena cava of a kidney donor; then perfusion of the left kidney with blood from the recipient is accomplished by transferring a clamp on aorta and vena cava from a position below to one above the left renal pedicle. Sprague-Dawley rats weighing around 200 g were matched according to weight and sex. The recipient was nephrectomized 18-24 hours prior to test, to increase its sensitivity to renal pressor substances. Following anesthesia with Amytal (9 mg/100 g of body weight), inguinal and neck areas were dissected for vascular anastomoses, tracheal cannulation and blood pressure recording. While the donor was anesthetized and prepared for transplantation, blood was withdrawn from the recipient through a femoral cannula to bring and maintain blood pressure at levels below 50 mm Hg. Then blood circulation was established between the recipient rat and the graft. Blood pressure was recorded on a smoked drum with a small bore mercury manometer. Pressure curves obtained were compared with those from nephrectomized animals which either received a renal graft or were made hypotensive by bleeding.

Results. A. Effects of renal grafts on nephrectomized rats (Fig. 1A). Grafting of a normal kidney caused regularly a pressor response consisting of an initial sharp rise of about 10 mm Hg followed by a slight gradual increase and/or a plateau which was maintained for periods of observation up to 3 hours. B. Effects of hemorrhage on nephrectomized rats (Fig. 1B). Sham operation for grafting was performed in 9 nephrectomized rats which were subsequently subjected to hemorrhagic hypotension. Amounts of blood necessary to cause sustained hypotension below 50 mm Hg ranged from 1.8 to 3.6 ml per 100 g of body weight. Blood was withdrawn slowly, first to reach a pressure around 40 mm Hg then smaller amounts were collected until pressure stabilized. Following this procedure there was usually a subsequent small rise in pressure of about 10 mm Hg then a plateau and finally terminal collapse. Death occurred between 15 and 110 minutes after the last bleeding. Survival periods following stabilization of pressure around 40 mm Hg were not closely related to a definite volume of blood. Thus in a 212 g rat removal of 6.4 ml of blood caused a fall in pressure from 110 to 40 mm Hg and death within 104 minutes while in another rat weighing 220 g five ml of blood caused a fall from 108 to 30 mm Hg and death within 48 minutes. C. Effects of renal grafts on shocked nephrecto-

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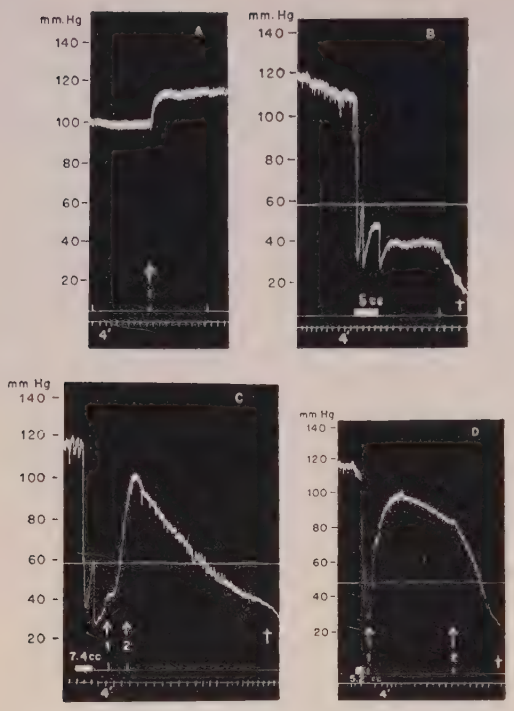

FIG. 1. Pressure responses in nephrectomized rats (A) to a renal graft, (B) to hemorrhage, (C) and (D) to renal grafts following hemorrhage. 1. grafting; 2, removal of graft.

mized rats. In 15 rats prepared as above, normal kidneys were grafted during established hypotension. There was an immediate and spectacular rise in pressure varying from 48 to 70 mm Hg towards levels existing prior to hemorrhage. This was followed by a prolonged plateau then a gradual and terminal fall. Survival periods varied between 26 and 108 minutes. Removal of the graft when the pressor response had reached its peak (Fig. 1C) or later on during the plateau (Fig. 1D) immediately caused a gradual and fatal circulatory collapse.

Discussion. It should be first emphasized that shock is a phenomenon difficult to standardize and that defense mechanisms are so varied and complex that a single factor may not have a significant influence on the evolution of the whole syndrome while having a definite beneficial effect on a particular function. The present experiments demonstrate clearly that the kidney restores blood pressure to near normal levels but has questionable effects on survival. The stimulus responsible for renin release is not anoxemia, but as suggested the low pressure acting on some renal baro-sensitive devices(6). Maintenance of blood pressure at a high level is only temporary. Its failure may be the result of an exhaustion of plasma renin-substrate or of loss of vascular reactivity(4,7).

Summary. Grafting of a normal kidney without interruption of renal circulation onto a nephrectomized rat during hemorrhagic hypotension causes a prompt return of blood pressure to near normal levels demonstrating that humoral renal mechanisms play a role in homeostatic regulation of blood pressure.

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Spontaneous Mineral Deposition in Sponge Biopsy Connective Tissue.* (26323)

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Adequate amounts of homogeneous connective tissue are unobtainable for most electrolyte metabolism studies. Tendon, skin and cartilage are heterogeneous structures with variation in electrolyte composition from one region to another, and pooling of samples often is required to supply sufficient material for quantitative analysis. Introduction of polyvinyl alcohol sponges beneath the dorsal skin of rats, guinea pigs and rabbits is followed by ingrowth of an essentially reproducible and relatively non-inflammatory connective tissue(1). The suitability of this experimental system for study of connective tissue electrolytes is herein evaluated.

Methods. Employing the technic of Boucek and Noble(1), Ivalon† polyvinyl alcohol sponges and other plastics were implanted in 50 Sprague-Dawley rats weighing 180-240 g each, including both sexes. Granulomas, harvested at 40 and 90 days, were weighed, dried overnight in an oven at 110°C, extracted with ether and acetone, and ashed in a muffle furnace at 700°C for 36 hours. The ash was dissolved in hydrochloric acid and subjected to the following analyses: 1) sodium and potassium, using the Baird flame photometer with internal standards(2); 2) calcium, by titration with potassium permanganate after precipitation with ammonium oxalate(3); and 3) phosphorus by the method of Fiske and Subbarow(4). Determinations were run in duplicate, usually agreeing within 3%. Subsequent to fixation of tissues for 24 hours in 10% neutral formalin and conventional processing, sections were cut at 6 μ thickness, mounted, stained by a Harris hematoxylin eosin sequence and toluidine blue. Fresh frozen sponge tissue was treated in an absolute alcohol-formalin xylol sequence, cut at 6 μ, mounted on quartz slides and microincinerated. Histochemical identification of inorganic phosphate was made by the Von Kossa staining procedure applied to absolute alcohol-fixed tissue. Polyvinyl alcohol sponge, soaked in silicone-resin‡ and dried overnight, as well as 3 additional plastics were implanted in separate groups of animals (Table II); the resulting tissue ingrowths were analyzed histologically as above.

Results. The high mean calcium and phosphorus content of the sponge tissue is apparent (Table I). Calcium to phosphorus ratios in 40 and 90-day sponges were 2.15 and 2.37 respectively—slightly above the limit expected if the salt were hydroxyapatite(5). "Bone salt" content approximately doubled in the period from 40 to 90 days. Two sponges indicated a 3- to 4-fold further rise of calcium and phosphorus content after 6 months of implantation. Another sponge, removed after one year, had become a solid block of mineral deposits. Sodium and potassium levels in the 40-day sponge were similar to those of other dense connective tissues (Table I)(6), and did not alter in the 90-day sponge, except for an insignificant decrease of sodium.

Histologic sections from 40 and 90-day sponges revealed a dense ingrowth of homogeneous connective tissue sparsely populated with fibroblasts, but lacking signs of inflammation. There was no evidence of systemic or local infection. Bluish granules in the hematoxylin-stained tissue were observed along the plastic margin in some sections. However, Von Kossa stains indicated a strongly positive dark granular band along the entire plastic-tissue interface of all 60 slides studied. An artifact related to fixation technic could not have caused this phenome-

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TABLE I. Electrolyte Content of Sponge Biopsy Connective Tissue.*

Tissue		Calcium	Phosphorus	Sodium	Potassium
		mM		meq	
40-day sponge	Mean	594	276.0	73.2	5.28
n = 9	S.E.	67.8	40.8	4.26	.78
	95% C.L.	438–750	182–369	63.3–83.1	3.42–7.14
90-day sponge	Mean	1224	516	60.6	4.68
n = 12	S.E.	153	42.3	3.00	.51
	95% C.L.	888–1560	423–609	54.0–67.2	3.54–5.82

* Per kg wet tissue. Data calculated on basis of fat-free dry tissue showed no greater homogeneity.

non inasmuch as microincineration studies of 40-day sponges confirmed the presence of a dense collection of inorganic material distributed along the plastic margin. Also, microincinerated and Von Kossa-stained sections from 4 unimplanted sponges were negative before and after incubation at 5°C for 48 hours in normal rat serum. In contrast to findings of Rubin and Howard(7), who studied a variety of pathologic calcifications, no metachromasia by toluidine blue staining was found in either the 40 or 90-day granulomas after removal of mineral deposits with dilute acid.

Other plastics evaluated in regard to deposit of mineral salts were studied at an early phase of tissue ingrowth. After 14 days implantation, polyvinyl alcohol sponges reveal a peripheral invasion by connective tissue for a distance of approximately 3-4 mm (Table II). Von Kossa stains were positive along the plastic margin only where contiguous with new cellular tissue (Fig. 1). Central region of the sponge containing lymph had no trace of Von Kossa staining. The silicone-coated sponges showed a similar ingrowth of tissue, but the Von Kossa staining was dense along the outside margin of the sponge, whereas little staining appeared along the invading tissue margins. The polyurethane, cellulose acetate sponges and polyethylene sheets induced a slight marginal reaction by the Von Kossa method. Of these plastics, only cellulose acetate induced granulomas comparable to those in polyvinyl alcohol sponge implants, but in contrast to the latter, showed a considerable polymorphonuclear leucocyte infiltration and local edema.

Discussion. As a model system for studying electrolytes in dense connective tissue, the sponge biopsy deviates from the ideal because of the deposition of "bone salt" accumulating at the plastic surface. Hydroxyapatite is believed to be the principal component of the lining material, inasmuch as the calcium and phosphorus content is high and hydroxyapa-

TABLE II. Histologic Studies of Plastic Implants under Dorsal Skin of Rats.*

Plastic	Duration of implant (days)	Tissue ingrowth (mm)	Von Kossa stain
Polyvinyl alcohol (sponge)	14	3–4	Strongly positive reaction distributed along entire plastic-tissue interface.
Polyvinyl alcohol (silicone coated sponge)	14	3–4	Moderately positive reaction only in scattered peripheral surface areas of sponge.
Cellulose acetate (sponge)	14	3	Moderate positive reaction in scattered peripheral surface areas of the plastic. No positive reaction seen in new granulation tissue.
Polyurethane (sponge)	14	.5–1	Negative reaction.
Polyethylene (sheets)	14 and 50	Layer of tissue (.25 mm thick)	*Idem.*

* No. of implants = 4 for each plastic in 10 rats.

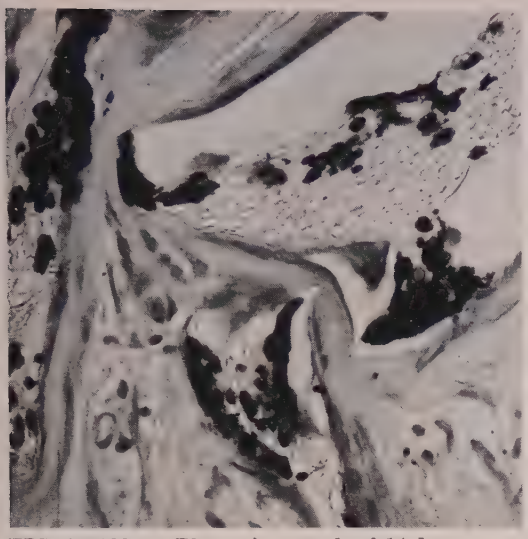

FIG. 1. 132×. Photomicrograph of 14-day sponge implant. Note Von Kossa positive staining granules confined to tissue-plastic interface.

tite is the prevailing salt found in mammalian tissues. Sodium and potassium content of the connective tissue as well as its histologic appearance was similar to that of other dense connective tissues, such as corium and tendon (8).

"Bone salt" accumulation could not be accounted for in the present experiments. Polyvinyl alcohol§ is a linear polymer with hydroxyl groups as the only regions of the molecule which would seem possible sites for nucleation of inorganic salts from ions in the adjacent lymph. In regard to these phases (9), interactions such as enzymatic esterification of hydroxyl groups with phosphate, or linkage of the plastic with calcium, phosphate or preformed hydroxyapatite are possibilities which have not been investigated. The data in Table II suggest that salt formation of the type demonstrated in this study requires the presence of living tissue adjacent to the plastic. Possibly an enzymatic reaction or other local conditions set by the presence of cells, fibers or their associated matrix is needed to induce the "bone salt" deposits. Decrease of the mineral deposition in silicone-coated sponges and failure of mineral deposition to occur in other plastics favor the view that polyvinyl alcohol engenders this phenomenon with some degree of specificity. The current data suggest that surgical use of polyvinyl alcohol sponges as pliable prosthetic devices in repair of heart valves, septal perforations and cosmetic defects, might show evidence eventually of significant mineral deposits (10).

§ Polymerized with formaldehyde.

Summary. Calcium and phosphorus content of connective tissue grown in rats implanted with polyvinyl alcohol sponges for 40 and 90 days was at high levels, which when correlated with histologic findings were indicative of salt formation. It appears that polyvinyl alcohol sponge has a greater tendency, from short term *in vivo* observations, to induce mineral deposits along its surfaces than other plastics tested. The data indicate a potential disadvantage to the usage of this plastic as a pliable prosthesis in human surgery.

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Virus Infection of Cells in Mitosis II. Measles Virus Infection of Mitotic HEp-2 Cells.* (26324)

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During studies of Human Epidermoid Carcinoma No. 2 Cells (HEp-2) infected with measles virus and stained with fluorescent antibody or acridine orange, we found a number of mitotic cells containing measles antigen. In addition, mitotic figures were present within virus induced syncytia. The experimental data reported here demonstrate that the virus antigen in the mitotic cells resulted from virus multiplication and that mitotic cells are readily recruited into syncytia induced by measles virus.

Materials and methods. The Edmondston strain of measles virus was passaged 23 times in HEp-2 cells prior to these experiments. HEp-2 coverslip cultures were prepared in Leighton tubes seeded with 60,000-80,000 cells suspended in tissue culture fluid consisting of 10% calf serum in mixture 199. Infected coverslip cultures were maintained in tissue culture fluid consisting of 1 to 10% calf serum in mixture 199, as indicated in text. Generally, syncytia formed more readily if infected HEp-2 cells were maintained with media containing high serum concentrations.

Acridine orange staining of coverslips was performed by the method of Armstrong and Niven(1). Fluorescent antibody was prepared from a pool of human immune gamma globulin selected for its high content of measles neutralizing antibody and conjugated to fluorescein isothiocyanate by the method described by Marshall(2). For better resolution of cellular detail, the coverslip preparations stained with fluorescein labeled gamma globulin were counterstained with Lissamine rhodamine (RB-200) conjugated with bovine albumin(3).

Localization of measles antigen in mitotic cells. Infectious tissue culture fluid (ITCF) was cleared of cell debris and clumps of virus by ultracentrifugation in a density gradient designed to minimize resuspension of pelleted material by diffusion or handling. Four-ml volumes of undiluted ITCF (2% calf serum in mixture 199) titering $10^{5.2}$ $TCID_{50}$ per ml were carefully layered into lusteroid tubes over 2 layers of 0.5 ml of sucrose, 21% w/w and 37% w/w, respectively. The tubes were then centrifuged for 2 hours at 35,000 rpm in a SW-39 Rotor driven by a Spinco Model L Ultracentrifuge. At the end of the ultracentrifugation the top 3 ml from 2 tubes were pooled, diluted 5-fold in mixture 199 with 1% calf serum and added in 1-ml volumes to 27 coverslip cultures of HEp-2 cells. The top 3 ml from a third tube, which contained "used" tissue culture fluid from an uninfected culture, was similarly diluted and added to 12 coverslip cultures, which served as controls. At intervals of 8, 22, 52 and 73 hours after incubation at 37°C, 7 infected and 3 control coverslip cultures were fixed in cold (–60°C) ethyl alcohol and stained with fluorescein labeled antibody.

Thorough examination of all control cultures exposed for 8 hours to the supernatant from ITCF did not show any yellow-green fluorescence. Specific fluorescence was first detected in an average of 30 well dispersed cells per coverslip in cultures fixed 22 hours after infection. The number of infected mitotic cells in each coverslip varied from one to 4. These were in metaphase, anaphase or telophase; infected prophase cells were not seen.

The appearance and site of localization of measles antigen in HEp-2 cells at rest have been described(4). Generally, we found that use of rhodamine counterstain permitted better visualization of cellular detail after staining with fluorescein labeled antibody. In our studies virus antigen was frequently present in both nucleus and cytoplasm of cells in in-

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terphase. In the nucleus, virus antigen appeared as dispersed small spherical granules distinct from nucleoli (Fig. 1). Virus antigen in cytoplasm of interphase cells was usually seen as a diffuse powdery fluorescence; less frequently, areas of specific fluorescence took the form of elongated or oval bodies arranged in a circle or semi-circle around the nuclei. In mitotic cells, virus antigen occasionally was present in the form of several small fluorescent granules adjacent to chromosomes but more frequently just under the cell membrane (Fig. 2-4). In general, mitotic cells were readily differentiated from interphase cells by their size and shape. In addition, chromosomes, which did not retain the rhodamine counterstain, were sharply silhouetted against the intense red-orange fluorescence of cytoplasm.

Coverslip cultures fixed and stained at 52 and 73 hours after infection showed an increased number of infected cells. In both groups, but particularly in coverslip cultures fixed at 73 hours, there were small syncytia and numerous foci of infection each consisting of 2-10 cells showing specific fluorescence. It is of interest to note that in some syncytia containing 5-10 nuclei, only 1 or 2 of these nuclei showed specific fluorescence. Similarly, virus antigen present in the cytoplasm appeared to be near some, but not all, nuclei contained in the syncytium. Infected cells in metaphase, and less frequently in anaphase and telophase, were seen in both sets of cultures. In 3 coverslip cultures fixed at 52 hours after inoculation, infected mitotic cells constituted 2.4, 5 and 8%, respectively, of total infected cells.

Recruitment of mitotic cells into syncytia induced by measles virus. Twenty-four hours after seeding with HEp-2 cells, 18 coverslip cultures were each exposed to 100 $TCID_{50}$ of measles virus suspended in mixture 199 with 1% calf serum and incubated for 72 hours. The medium in 6 coverslip cultures was then replenished with 10% calf serum in mixture 199; the remainder received 10^{-7} M colchicine (Nutritional Biochemical Co., Cleveland, Ohio) in the same medium.

Examination of coverslip cultures stained with acridine orange 96 hours after infection revealed the presence in both colchicine treated and untreated cultures of abundant syncytia containing as many as 20 nuclei each. In cultures without colchicine 3 of 68 syncytia contained a dense mass of material with the characteristic bright yellow fluorescence of DNA. This mass appeared to be similar in size and color to the chromosomes of uninfected cells in metaphase, but more irregular in shape. Nearly 70% of syncytia counted in infected cultures treated with colchicine contained at least one, but usually several such masses (Fig. 5). In these syncytia some masses appeared to be identical in size and shape to aggregated chromosomes of cells in metaphase seen in untreated cultures. Others were smaller, more irregular in shape, and appeared to be amorphous clumps of chromosomes.

These studies on recruitment of mitotic cells into infected syncytia were repeated several times with variations in the time at which the cultures were fixed and stained. In one experiment, colchicine was withdrawn after 24 hours of exposure. In another experiment, uninfected cells arrested in metaphase by colchicine were seeded among cells in previously infected cultures. In these latter experiments, it was again evident that treatment with colchicine markedly increased the number of mitotic cells recruited into infected syncytia.

Discussion. The conclusion that measles antigen present in mitotic cells resulted from virus multiplication is based on the following evidence: (1) Ultracentrifugation served to clear the inoculum of cell debris, clumps of virus antigen, and most of the virus. The absence of specific fluorescence in cells exposed to the inoculum for 8 hours supports the contention that virus antigen was formed *de novo* following infection of cells in the coverslip cultures. (2) It seems unlikely that measles virus antigen was merely released from degenerated cells and subsequently ingested by mitotic cells. At 22 hours after infection very few cells (average of 30 in each coverslip culture) exhibited specific fluorescence, but those that did contained as many as 6 discrete fluorescent granules distributed

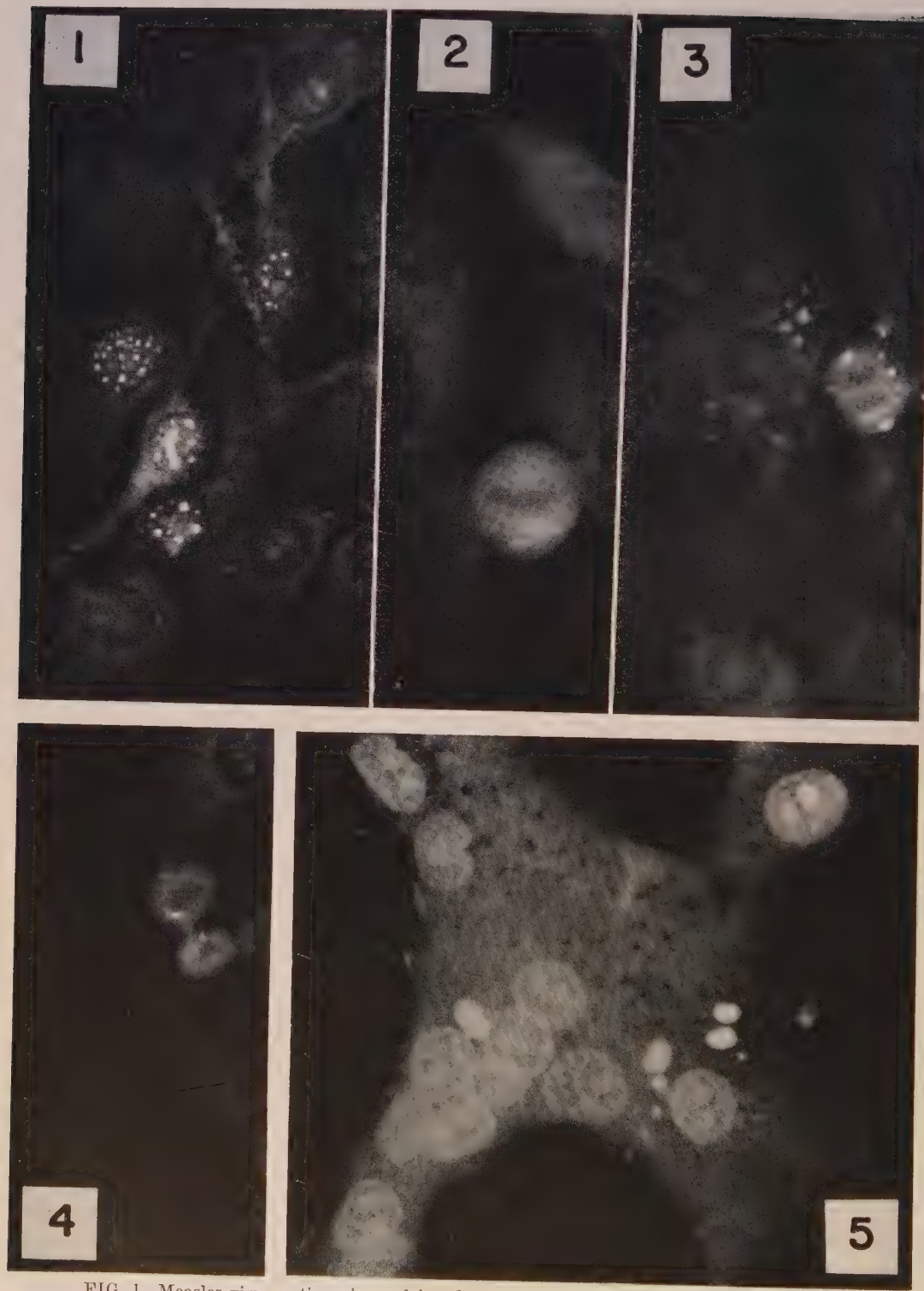

FIG. 1. Measles virus antigen in nuclei and cytoplasm of interphase cells. 480×. Fluores-

cein labeled antibody stain, counterstained with Lissamine rhodamine conjugated to bovine albumin.

FIG. 2. Granules of measles virus antigen in metaphase cell. 720×. Fluorescein labeled antibody stain, counterstained with Lissamine rhodamine conjugated to bovine albumin. The unstained chromosomes are silhouetted against the intense red-orange rhodamine fluorescence of the cytoplasm.

FIG. 3. Granules of measles virus antigen in late anaphase or telophase cells. A portion of a syncytium of HEp-2 cells is also shown. 480×. Fluorescein labeled antibody stain, counterstained with Lissamine rhodamine conjugated to bovine albumin.

FIG. 4. Measles virus antigen in daughter cells (late telophase or early interphase). Note that virus antigen is present in both daughter cells. 480×. Fluorescein labeled antibody stain, counterstained with Lissamine rhodamine conjugated to bovine albumin.

FIG. 5. Mitotic figures and clumps of chromosomes (in the photomicrograph they appear as white oval bodies without internal structure) in syncytium of HEp-2 cells induced by measles virus. 480×. Stained with acridine orange.

in their cytoplasm. It is our contention that it is unlikely that so many granules could be ingested by a single cell in a culture in which very few cells showed evidence of infection unless the mitotic cells ingested whole infected cells. Careful examination failed to reveal additional nuclei or other evidence that a whole cell was ingested. Nevertheless, demonstration of measles antigen in mitotic cells is not by itself evidence of mitotic division of infected cells. The data are also consistent with the hypothesis that a cell may become infected at any stage in the mitotic cycle (including interphase), and that infection causes mitotic arrest.

It is of interest that very different results were obtained in experiments with the MP strain of Herpes simplex virus, as described elsewhere(5). In these studies, HEp-2 cells in prophase appeared to be more susceptible to recruitment into syncytia induced by Herpes simplex virus than were cells in metaphase; infected cells in anaphase and telophase were not seen. Mitotic cells arrested in metaphase by colchicine were also resistant to infection. However, these cells readily fused to form giant cells after withdrawal of the drug. From comparisons of HEp-2 cell cultures infected with measles and Herpes simplex virus, it seems reasonable to postulate that (1) susceptibility of HEp-2 cells to virus infection may vary depending on their stage in the mitotic cycle and, (2) susceptibility to infection of cells at each mitotic stage may vary from one virus to another.

Summary. Fluorescein labeled antibody staining of HEp-2 cell cultures infected with an inoculum of measles virus free of cell debris and other particulate matter revealed the presence of measles virus antigen in cells in metaphase, anaphase and telophase. Mitotic cells were shown to be readily recruited into syncytia induced by measles virus, both in absence and presence of colchicine. These observations contrast with the results of similar studies made in HEp-2 cell cultures infected with Herpes simplex virus and indicate that cells in different stages of the mitotic cycle are not uniformly susceptible to infection with different viruses, or even the same virus.

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In vitro Effect of Acetylcholine on Adrenocortical Secretion of Incubated Rat Adrenals.* (26325)

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Acetylcholine (ACh) appears capable of stimulating adrenocortical secretion both *in vivo* and *in vitro*. *In vivo* evidence suggests increased cortical function following parenteral administration of ACh(1) or following splanchnic nerve stimulation wherein increased quantities of ACh might be assumed to be released(2,3). Rosenfeld(4) has demonstrated direct stimulation of corticosteroidogenesis in perfused calf adrenals by ACh and other cholinergic drugs as evidenced by increased corticoid production and atropine inhibition of this effect. Results of these studies indicate, furthermore, that ACh stimulation of corticosteroidogenesis may be mediated *via* regulation of 17-hydroxylation as indicated chromatographically by a primary increase in 17-hydroxylated corticoids. The present investigation has explored possible species differences in *in vitro* stimulation of adrenocortical secretion by ACh which might be linked to its action in regulating 17-hydroxylation. We have used an indirect approach to the problem employing the incubated rat adrenal which is known to secrete primarily corticosterone(5) and in which 17-hydroxylation probably does not occur.

Methods. Sprague-Dawley CD (caesarean delivered) or Wistar male albino rats weighing 150-300 g each and maintained on tap water and Purina chow *ad libitum* were used. Both adrenals were removed under sodium pentobarbital anaesthesia, trimmed of periadrenal tissue, quartered or cut into sixths, and the pieces distributed equally among 4 to 6 sectors in a Petri dish humidor kept on ice. Thus, each sector received a representative piece of adrenal from each rat. Pooled adrenal tissue from each sector at least equivalent in weight to one adrenal was weighed on a torsion microbalance and transferred to a vessel containing 2.0 ml ice-cold Krebs - Ringer - bicarbonate - glucose medium (pH 7.4). Vessels were flushed for 10 minutes with 95% O_2—5% CO_2, sealed, and incubated at 38°C in a Dubnoff metabolic incubator. After pre-incubation for 30 minutes, incubation for 2 hours was carried out in fresh medium with adrenocorticotropin (ACTH, Armour ACTHar, LalA, Blend 2H, 1.39 ± 0.12 I.U./mg) in maximally effective doses (500 mμ/100 mg tissue) added to one flask of each series, and ACh bromide or chloride (Eastman organic chemicals, Lot Nos. 26A and 374245) in varying doses added to other flasks. In these series one flask received no additive. In one series acetylcholinesterase (AChase) (Sigma Chemicals Lot. No. 28-059) was added at variable time intervals following initial stimulation with effective doses of either ACTH or ACh. Upon completion of incubation, the medium was extracted with methylene dichloride, duplicate measurements made on unpurified, dried extract residues with blue tetrazolium (BT) and by measurement of peak absorption in the ultraviolet at 240 mμ (UV), and output rates calculated as μg Corticosterone equivalents/100 mg adrenal (wet wt)/2 hours incubation as previously described(6). ACTH, ACh, or AChase did not contribute significantly to BT or UV values.

Results. Stimulation of adrenocortical output by ACh bromide in doses ranging from 0.0001 to 10,000 μg/100 mg adrenal was investigated using Sprague-Dawley rat adrenals. Table I shows that a maximally effective dose of ACTH significantly increases adrenal output as measured both by BT and UV methods, whereas ACh at all doses tested above 0.0001 μg/100 mg adrenal apparently increases only output of BT reducing substances. Furthermore, the magnitude of increased output achieved with a maximally effective dose of ACh is only approximately

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TABLE I. Adrenocortical Secretion of Incubated Male Sprague-Dawley (CD) Rat Adrenals in Presence of ACh Bromide or ACTH.

Additive	Dose/100 mg adrenal	BT μg/100 mg/2 hr	BT Change μg/100 mg/2 hr	BT Change %	UV μg/100 mg/2 hr	UV Change μg/100 mg/2 hr	UV Change %
None		10.02 ± 4.30* (10)†			20.84 ± 4.10		
ACTH	500 mu	39.30 ±13.22 (12)	+29.28	+292	52.53 ±15.24	+31.69	+152
ACh	.0001 μg	8.44 ± 2.84 (6)	− 1.58	− 15.7	16.30 ± 4.31	− 4.54	− 21.7
"	.01 "	13.40 ± 3.14 (7)	+ 3.38	+ 33.7	19.40 ± 4.47	− 1.44	− 6.89
"	.1 "	15.43 ± .83 (4)	+ 5.41	+ 54.0	14.72 ± 9.38	− 6.12	− 27.3
"	1 "	16.10 ± 6.26 (4)	+ 6.08	+ 60.6	22.90 ± 5.32	+ 2.06	+ 9.84
"	1000 "	16.83 ±10.81 (5)	+ 6.81	+ 67.9	17.16 ± 7.74	− 3.68	− 17.6
"	10,000 "	15.80 ± 1.08 (4)	+ 5.78	+ 57.6	22.04 ± 3.72	+ 1.20	+ 5.74

* Mean ± S.D. † No. of experiments.

1/5 that achieved with ACTH. Although ACh at a dose of 0.0001 μg/100 mg adrenal does not increase adrenal output of BT reducing substance, a relationship apparently exists between dose and response at levels between 0.01 to 1000 μg/100 mg adrenal with the minimal dose requisite for maximal response between 1 and 1000 μg/mg adrenal.

To determine whether adrenocortical stimulation by ACh might be related to strain of rat or to ACh derivative, investigations were carried out using Wistar rat adrenals and ACh chloride. For comparison ACh bromide and ACTH were tested simultaneously. Two doses of ACh were tested, one known to produce measurable but submaximal stimulation and the other maximal stimulation of BT reducing substances. The data in Table II compared to those of Table I illustrate similar findings with respect to ACTH and ACh effects on adrenal output regardless of ACh derivative or strain of rat used. The

TABLE II. Comparison of *In Vitro* Effect of ACh Bromide, ACh Chloride and ACTH on Adrenocortical Secretion of Male Wistar Rat Adrenals.

Additive	Dose/100 mg adrenal	BT μg/100 mg/2 hr	BT Change μg/100 mg/2 hr	BT Change %	UV μg/100 mg/2 hr	UV Change μg/100 mg/2 hr	UV Change %
None		8.65 ± 2.23* (3)†			9.20 ± 4.89		
ACTH	500 mu	43.07 ± 5.65 (3)	34.42	+398	52.2 ± 9.11	43.00	+468
ACh Br	1000 μg	16.00 ± 2.82 (4)	7.35	+ 85.0	11.36 ± 9.41	2.16	+ 23.5
Idem	.1 "	12.95 ± 3.74 (4)	4.30	+ 49.8	8.41 ± 5.38	− .79	− 8.54
ACh Cl	1000 "	12.00 (1)	3.35	+ 38.8	7.66	−1.54	− 16.8
Idem	.1 "	10.90 (1)	2.25	+ 26.0	11.30	+2.10	+ 22.9

* Mean ± S.D. † No. of experiments.

TABLE III. *In Vitro* Effect of Acetylcholinesterase on Adrenocortical Secretion of Male Sprague-Dawley (CD) Rat Adrenals Following Initial Stimulation with ACh Bromide or ACTH.

Additive	Dose/100 mg adrenal	BT μg/100 mg/2 hr	BT Change μg/100 mg/2 hr	BT Change %	UV μg/100 mg/2 hr	UV Change μg/100 mg/2 hr	UV Change %
None		10.02 ± 4.30* (10)†			20.84 ± 4.10		
ACTH	500 mu	48.70 ±21.11	+36.68	+366	65.30 ±28.72	+44.46	+213
+ AChase‡	1000 μg						
ACh	1 μg	6.11 ± 4.35	− 3.91	− 39.0	10.78 ± 4.89	−10.04	− 48.2
+ AChase‡	2 "	(5)					
ACh	1 "	14.40 ± 2.02	+ 4.38	+ 43.7	22.27 ± 2.65	+ 1.43	+ 6.86
+ AChase§	2 "	(4)					
ACh	1 "	16.10 ± 6.26 (4)	+ 6.08	+ 60.6	22.90 ± 5.32	+ 2.06	+ 9.84
AChase	2 "	8.05 ± 3.13 (4)	− 1.97	− 19.6	18.62 ± 2.64	− 2.22	− 10.7

* Mean ± S.D. † No. of experiments. ‡ Added 30 min. after initial stimulation.
§ Added 60 min. after initial stimulation.

lower BT response obtained with ACh chloride as compared to ACh bromide cannot be explained. This applies as well to the apparently significant UV response observed with the low dose of ACh chloride.

To demonstrate whether increased output of BT reducing substances is attributable directly to ACh, inhibition of this effect was attempted with AChase. In this series appropriate amounts of AChase (at least 2× the dose of ACTH or ACh) were added to the incubation medium at 30 or 60 minutes after initial addition of ACTH or ACh bromide in doses previously shown to be effective (Table I). The effect of AChase alone in doses comparable to those used to inhibit ACh was also tested in similar manner. Table III summarizes results which include for comparison average output rates in absence of additive or in the presence of ACh alone obtained previously under similar conditions. These data show that whereas AChase *per se* does not substantially alter adrenal BT output and does not inhibit ACTH, it does inhibit ACh. Addition of AChase 30 minutes after addition of ACh did not increase BT output, whereas AChase added after 60 minutes increased the output to a level below that following stimulation with ACh alone for 2 hours. This finding justifies the conclusion that the adrenal tissue was initially responsive to ACh and that AChase inhibition did occur.

Discussion. Our findings that ACh *in vitro* does not increase rat adrenal output of substances measurable by UV are in disagreement with those previously reported for perfused calf adrenals(4). This may reflect differences in species or in the *in vitro* methods employed. Demonstration by us that ACh stimulates release of BT reducing substances without concomitant increase in substances measurable by UV suggests that the former are not Δ^4-3 ketosteroids. Furthermore, it has been shown that adrenals of several mammalian species may release significant quantities of non-α ketolic BT reducing substances under the influence of serotonin(7). Although the BT substances released by ACh have not yet been identified, it is possible that they are not α ketol-Δ^4-3 ketosteroids. Since the rat adrenal presumably does not possess a 17-hydroxylating system it may be inferred that if ACh stimulates steroidogenesis it is primarily by regulating 17-hydroxylation as previously suggested. Our finding that ACh stimulation of BT reducing substances is inhibited by AChase further demonstrates that the effect is specific for ACh. Although it is not valid on the basis of these data to attribute a greater activity to the ACh bromide as compared to ACh chloride, our findings are highly suggestive.

Since adrenal output in presence of ACh is approximately 1/5 that achieved with ACTH and since AChase does not inhibit ACTH action, it would appear that ACTH action is not mediated *via* an ACh controlled mechanism.

Summary. The *in vitro* effect of acetylcholine on adrenocortical secretion of rat adrenals was investigated by direct methods of analysis. ACh increases rat adrenal output of BT reducing substances without corresponding increase in substances measurable by UV regardless of strain of rat or type of ACh derivative used. A relationship between dose and response was observed with ACh doses between 0.01 to 1000 μg/100 mg adrenal. Output achieved with a maximally effective dose of 1000 μg/100 mg adrenal was approximately 1/5 that observed following maximal ACTH stimulation. Specificity of the ACh effect was demonstrated by inhibition with acetylcholinesterase.

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Enzymic Hydrolysis of Stilbestrol Diphosphate *in vitro* and *in vivo.* (26326)

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Stilbestrol diphosphate is an inactive "transport" form of stilbestrol currently employed in treatment of prostatic cancer. By virtue of the high level of phosphatase activity in malignant prostatic tissue(1) active stilbestrol is presumed to be preferentially released within the tumor and its metastases(2, 3). The absence of gynecomastia and other feminizing effects in patients treated with stilbestrol diphosphate is adduced as further evidence for target specificity of the substance. Recent studies with C^{14}-labelled stilbestrol diphosphate in prostatic carcinoma patients have shown the occurrence of considerable localization of free stilbestrol in the prostate; no localization occurred when stilbestrol was administered(4). However, in view of the abundant distribution of acid and alkaline phosphatases in animal tissues it might be expected that the hydrolysis of stilbestrol diphosphate would not be restricted to prostatic tissues, malignant or otherwise. In fact, it has been reported(5), without supporting data, that the compound is dephosphorylated by various rat tissues and fresh human prostatic tissue. In the present study, the phosphatase activity of several rat tissues towards stilbestrol diphosphate has been examined. Also, an attempt has been made to ascertain the extent of this activity *in vivo* by measurement of stilbestrol plasma levels following administration of the disphosphate ester.

Materials and methods. Tissue preparations. Homogenates of rat spleen, prostate, kidney, liver and of brain were prepared by grinding the tissues in a Potter-Elvehjem type homogenizer with 0.154 M KCl in the proportion of 1:20. To 1.0 ml of homogenate was added 0.5 ml of 0.2 M buffer solution and the mixture placed in a water bath at 37° for 5 minutes; 0.5 ml of 4×10^{-3} M stilbestrol diphosphate (disodium salt), dissolved in the same buffer, was then added to start the reaction. Tris (hydroxymethyl) aminomethane buffer was used to provide a pH of 7.4; acetate for pH 5.0, and borate for pH 9.0. A tissue blank was run with each experimental vessel. After the desired incubation time, which varied from 5 minutes to 2 hours, 5 ml of 6% trichloracetic acid was

added to terminate the reaction, and the sample centrifuged. The orthophosphate content of the clear supernatant was determined by the method of Fiske and Subbarow(6). Phosphate content of the appropriate tissue blank in each case was subtracted from that of the experimental sample. The stilbestrol diphosphate stock solution was assayed and found to contain no determinable free phosphate or free stilbestrol.

Determination of stilbestrol in plasma. Dryer(7) has described a method for assay of stilbestrol in tissue extracts. Modifications were required for application of the method to blood plasma. Dichloromethane replaced the acetone-ether of Dryer's method for extraction of stilbestrol from blood plasma. The advantages of dichloromethane are that it yields recoveries of 98 ± 2.1%, provides solutions free of turbidity, and gives zero blank values.

Procedure. Shake the plasma sample (usually 2 ml) with 10 ml of dichloromethane in a separatory funnel for 3 minutes. Transfer the organic phase to a second funnel and extract with 2 ml of 2.5N sodium hydroxide to remove the free stilbestrol. Separate the 2 phases, and acidify the aqueous phase with 2 ml of 5N sulfuric acid and extract with 10 ml of dichloromethane as before. Transfer the latter phase to a 20 × 170 mm Pyrex test tube and evaporate to dryness over a steam bath. Dissolve the dried residue in 1 ml of glacial acetic acid, add 0.1 ml of bromine reagent (1% v/v in glacial acetic acid) and place the tube in boiling water for exactly 2 minutes. Cool the tube, add 5 ml of 40% ethanol, mix, and read the optical density after 1 min at 500 mμ (Bausch & Lomb Spectronic 20) against a blank prepared by carrying a stilbestrol-free plasma sample through the whole procedure. The optical density measured at 500 mμ is a linear function of the plasma stilbestrol content within the range 0-25 μg. Reproducibility was found to be 1.2% at the 25 μg level and 6.5% at the 2 μg level.

Materials. Stilbestrol diphosphate (Honvol, Horner), a sterile aqueous solution of the disodium salt, was used for intravenous administration to rabbits. N-Acetyl-p-aminophenol (Atasol, Horner) 250 mg/kg body weight, was given by stomach tube as a suspension in tap water.

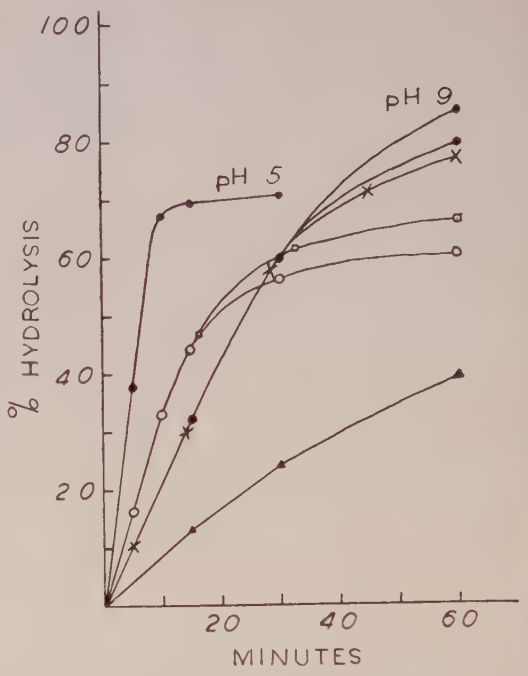

FIG. 1. Hydrolysis of stilbestrol diphosphate by rat tissue homogenates; pH 7.4, unless otherwise indicated. ● prostate, ○ liver, □ kidney, × spleen, ▲ brain.

Results and discussion. The hydrolysis of stilbestrol diphosphate by homogenates of several different rat tissues was measured at pH 7.4; hydrolytic activity of prostate tissue was studied further at pH 5 and 9. Results are presented in Fig. 1. It can be seen that the ester is readily dephosphorylated by prostate, spleen, kidney, liver and brain tissues. At pH 7.4 liver and kidney show the same rate of phosphatase activity, which is slightly greater than that of prostate and spleen. At might be expected, the rate of hydrolysis by prostatic phosphatase was greatly increased by lowering the pH to 5.0, while increasing the pH to 9.0 failed to influence the rate. The results also indicate that hydrolysis proceeds beyond the monophosphate stage but not to complete dephosphorylation; this can be clearly seen if the incubation is continued to 2 hours.

Erythrocytes of several animal species, in-

TABLE I. Plasma Levels of Stilbestrol in Rabbits Following a Single Intravenous Injection of Stilbestrol Diphosphate (92 mg/kg Body wt).

Min. after dosage	Plasma stilbestrol, μg/ml*
15	16.3
30	9.1
45	3.9
60	2.5
90	2.1
120	1.2

* Avg values from 3 rabbits, except for 60 and 120 min. intervals which are avg values from 6 rabbits.

cluding the human(8) show appreciable acid phosphatase activity. It was of interest, therefore, to test the behavior of red cell phosphatase towards stilbestrol diphosphate. The latter at 10^{-3} M concentration was incubated with whole rabbit blood at 37°C. After 10, 20 and 30 minutes of incubation it was found that 12.6, 17.3 and 22.4%, respectively, of the ester was hydrolyzed. When the same amount of stilbestrol diphosphate was incubated with plasma for 30 minutes, only 5% was hydrolyzed. Thus the red cells, and probably to some extent the leukocytes, account for most of the phosphatase activity of whole rabbit blood toward stilbestrol diphosphate.

As an indication of hydrolysis of the ester *in vivo,* stilbestrol diphosphate (92 mg/kg body wt) was injected intravenously into rabbits over a period of 2-3 minutes, and blood plasma was assayed chemically for free stilbestrol. It was found that free stilbestrol appeared in the blood stream as early as 5 minutes after termination of injection, and within 15 minutes had reached a concentration of 16.3 μg/ml of plasma (Table I). This was followed by a rapid decline in concentration, only 1.2 μg/ml remaining after 2 hours. Plasma levels of free stilbestrol were greatly increased by oral administration of a competitive glucuronylation inhibitor, N-acetyl-p-aminophenol (250 mg/kg) one hour prior to injection of stilbestrol diphosphate (unpublished results). Since glucuronylation occurs predominantly in the liver, the marked effect of the glucuronylation inhibitor probably indicates that a considerable amount of the stilbestrol diphosphate hydrolyzed in the liver was returned to the plasma as stilbestrol glucuronide. In this connection, stilbestrol monoglucuronide has been isolated from rabbit urine following subcutaneous injection of stilbestrol diphosphate(9). Studies in human subjects have shown the localization of stilbestrol in the prostate, liver, kidney, bone, and other tisues when stilbestrol diphosphate was administered(4,10,11). Hydrolysis of stilbestrol diphosphate by various tissues of the rabbit probably accounts for the presence of stilbestrol in the blood stream. Undoubtedly the acid phosphatases of the erythrocytes(8) also make a contribution.

Comment. The adult human prostate has up to 1000 times the acid phosphatase activity of that of other human tissues(12,13,14). However, total phosphatase activity of other body tissues combined, including blood, far outweigh that of the prostate and its malignancies. Although the localization of stilbestrol in prostatic tissue following intravenous administration of stilbestrol diphosphate has been demonstrated to occur(4,11), it is obvious that the ester would be hydrolyzed by other tissues as well. The high plasma levels of free stilbestrol observed in rabbits following injection of stilbestrol diphosphate tend to bear this out. It is probable therefore that stilbestrol diphosphate therapy of prostatic cancer allows for indirect effects of stilbestrol, for example, through the pituitary by inhibition of gonadotrophin secretion(15) as well as a direct cytotoxic action on prostatic tissue.

A notable feature of stilbestrol diphosphate therapy is the absence of gynecomastia(16), which has not been adequately explained. Considering the size of the individual doses employed in therapy (usually 500-1000 mg)(11,17), it is conceivable that the absence of gynecomastia may be due to presence of high concentrations of stilbestrol, which would tend to suppress the estrogenic stimulation of mammary gland growth. This biphasic action of estrogens is a well known phenomenon. Gardner(18) demonstrated that small doses of estrogen stimulated, while large doses inhibited mammary gland growth in mice and monkeys. Huggins *et al.*(19)

have shown that small amounts of estrogens stimulate growth of mammary tumors in ovariectomized rats, while large quantities block their growth.

Summary. Stilbestrol diphosphate is rapidly hydrolyzed by the phosphatases present in homogenates of rat prostate, liver, kidney and spleen, and to a lesser extent by brain tissue. A convenient chemical procedure is described for assay of free stilbestrol in plasma. Following administration of stilbestrol diphosphate to rabbits, appreciable amounts of free stilbestrol appear in the blood.

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Control of Plasma Non-Esterified Fatty Acids in Pregnancy and the Puerperium.* (26327)

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As term approaches the concentration of non-esterified fatty acids (NEFA) in peripheral blood plasma is significantly increased in normal human pregnancy. Prompt return toward normal non-pregnant values occurs within 48-72 hours after delivery. The NEFA fraction thus participates in the hyperlipidemia of pregnancy, although the physiological basis for the gestational increase in blood lipids remains unknown. We have suggested, however, that the lipid change may reflect altered carbohydrate utilization as another aspect of the diabetogenic influence of pregnancy(1).

The studies of Dole(2-3) and of Gordon (4-5) have related the plasma concentration of NEFA to carbohydrate utilization. Increase in plasma non-esterified fatty acids is observed when carbohydrate is either unavailable or cannot be utilized because of metabolic error. In either case, as Gordon has suggested, "caloric homeostasis" tends to be preserved due to the extremely rapid rate of NEFA utilization even at low plasma levels (5). Although in general NEFA release from fat depots to plasma reflects the prevailing level of carbohydrate utilization, the following observations relative to experimental modification of plasma NEFA in pregnancy and the puerperium suggest that control of fatty acid concentration in blood may be quite complex.

This complexity was first suspected when it was observed that degree of change in plasma NEFA after insulin treatment did not

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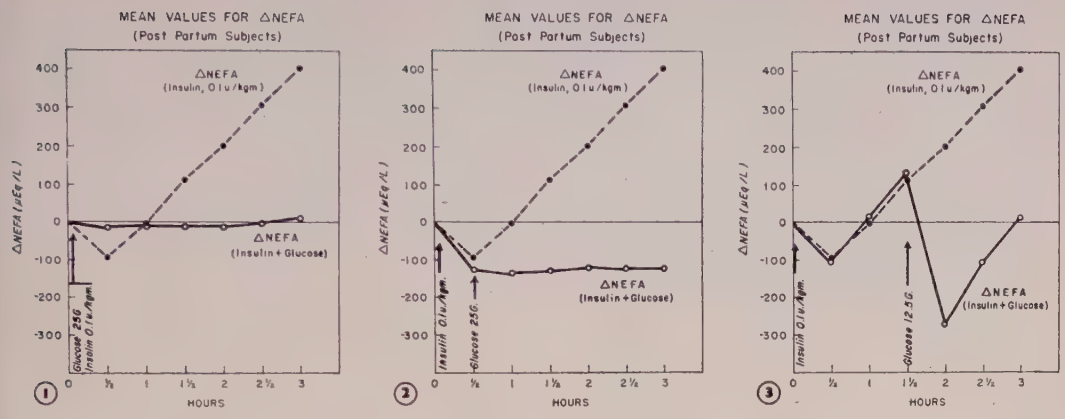

FIG. 1-3.

parallel development of insulin resistance in late pregnancy. Following a standard dosage of 0.1 U/kg regular insulin free of hyperglycemic-glycogenolytic factor, the decrease in plasma NEFA was essentially the same in non-pregnant and pregnant subjects despite significant attenuation of hypoglycemia and hypophosphatemia in the latter group(6). In the early puerperium (4th or 5th post partum day) even with recovery of insulin sensitivity, NEFA decrements were significantly less than observed in late pregnancy, degree of change in NEFA concentration departing from that of glucose.

Method. In the present investigation normal puerperal women were studied on the 4th or 5th post partum day after uncomplicated vaginal delivery. One group of 20 subjects were given by vein 0.1 unit of glucagon free regular insulin/kg body weight. A second group of 20 received with the insulin 25 g 50% glucose in water. A third group of 20 first were treated with insulin and then given 25 g glucose one half hour later. Finally a group of 20 puerperal subjects received 12.5 g glucose 1½ hours after the standard insulin dose. A limited number of other observations were made on pregnant and non-pregnant subjects with various combinations of insulin and glucose treatment. These data will be referred to but not constitute the basis of this report. Blood samples were obtained before insulin treatment and at half hourly intervals thereafter for 3 hours. After separation of formed elements by centrifugation NEFA was determined in duplicate on plasma aliquots by the method of Dole(2).

Results. A characteristic metabolic response in the early puerperium was the complete suppression of NEFA change when 25 g glucose was given I.V. with the insulin, although this amount of glucose was without effect on the level of fatty acids when given alone(7). This is shown in Fig. 1 where the NEFA mean values for 2 groups of 20 normal postpartum subjects are plotted. One group received insulin and responded by the typical biphasic change in NEFA concentration during the 3 hour period of observation. The other group received 25 g glucose with the insulin which resulted in a stabilizing effect on NEFA and gave almost quantitative inhibition of the biphasic response.

In Fig. 2 is shown the result of 25 g glucose given ½ hour after insulin administration. Attention is directed to the effect of this amount of glucose which suppressed recovery and "rebound" above fasting NEFA values. This effect was observed not only in the early puerperium but for all normal pregnant and non-pregnant subjects studied. If the glucose is given 1½ hours after the insulin plasma NEFA concentration becomes fixed or stabilized at the level attained at that time.

When a fraction of the 25 g glucose is given (*i.e.* 12.5 g) with the insulin the decrease in NEFA concentration is exaggerated. This was demonstrated for all categories of subjects studied whether pregnant, puerperal or non-pregnant. In contrast to the 25 g glucose load the 12.5 g glucose dosage did

not stabilize NEFA when given at ½ or 1½ hours after insulin. On the contrary, further abrupt decrements in NEFA were observed (Fig. 3).

Comment. It appears that in certain circumstances the amount of available carbohydrate can profoundly influence the effect of insulin on concentration of non-esterified fatty acids in peripheral blood plasma. Whether these effects are related to release or to utilization of NEFA is uncertain but the changes in NEFA concentration may depend upon the relative amount of carbohydrate presented to the tissues at various levels of available insulin from endogenous or exogenous sources. Recent studies suggest that glucose is metabolized preferentially by the peripheral tissues(8,9) and it is possible that the balance between fatty acid and glucose metabolism in fasted subjects receiving insulin is determined by the amount of carbohydrate available. Small amounts of glucose (*i.e.* 12.5 g) in presence of exogenous insulin or insulin of pancreatic origin resulting from glucose administration itself are insufficient to suppress NEFA utilization. This effect together with direct suppression of NEFA release from adipose tissue by insulin as shown by radioisotope studies(10) results in a net decrease in NEFA concentration. With a larger glucose load (25 g) perhaps a new balance is established with relatively less NEFA utilized because of the greater amount of carbohydrate available. In this situation NEFA concentration tends to "stabilize" at the point in our experiments when the glucose is given. The relative effects of insulin upon fatty acid synthesis and release may also bear on our data as well as the "recycling" of NEFA as described by Laurell(11) and by Fredrickson and Gordon(12).

Whatever factors are involved, the preliminary observations reported here suggest that plasma level of NEFA is subject to a regulatory mechanism that is dependent not only upon ability to metabolize carbohydrate and availability of insulin but upon quantity of carbohydrate present. It can be inferred also that plasma concentration of non-esterified fatty acids is not solely determined by the rate of release of these acids from fat depots.

Summary. The amount and timing of glucose administered to fasting post partum women receiving intravenous insulin determines the amount and direction of change in plasma NEFA. Twenty-five grams of glucose inhibits the characteristic response of NEFA to insulin while half this dosage either enhances the insulin effect or causes secondary decrements in non-esterified fatty acid. These results suggest that the regulation of plasma NEFA is a complex function that may involve fatty acid synthesis, utilization and recycling as well as controlled release from fat depots.

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Influence of *Pasteurella tularensis* on Enzymes Involved in Energy Metabolism in Tissues of Rats.* (26328)

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The observation that d-amino acid oxidase activity was suppressed markedly in livers and kidneys of tularemic rats(1) suggested the possibility that other enzyme systems, including those of the tricarboxylic cycle, might be influenced adversely during infection. Attention has been focused upon the activities of liver aconitase and liver fumarase which are among those that participate directly in energy metabolism, and which thus might be expected to reflect the influence of *Pasteurella tularensis* on fundamental metabolic mechanisms in its host.

Pinchot *et al.*(2) demonstrated that the effects of adrenal exhaustion occurring in tularemia could be somewhat alleviated by administration of cortisone. Their data have suggested the possibility that this is the result of temporary support of energy metabolism at nearly normal levels.

In view of their findings, studies also were conducted to determine the influence of administration of cortisone during the course of infection on rat liver aconitase and fumarase activity.

Materials and methods. Culture. Strain Sm of *P. tularensis*, which has a rat LD_{50} of approximately 3.5×10^3 organisms, was employed throughout this study. This culture was isolated in 1941 from a human ulcer by Dr. Lee Foshay, Univ. of Cincinnati College of Medicine, Cincinnati, Ohio.

Experimental methods. Aconitase and fumarase activity were determined according to the method of Racker(3). Cortisone acetate from Nutritional Biochemicals Corp., Cleveland, Ohio was administered in a mineral oil suspension by intraperitoneal injection.

The rats were maintained on a commercial high energy ration from Security Mills, Inc., Knoxville, Tenn. Test animals were fasted for 12 hours before each experiment but were allowed water *ad libitum*. Rats selected for inoculation received an infecting dose of one LD_{50}. These animals were killed by decapitation in appropriate numbers daily, following infection, for 4 days. After exsanguination, specimens of liver tissue were removed immediately to chilled buffer solution for preparation of homogenates.

All determinations were made in duplicate and enzyme activity was calculated on the basis of dry weight of the tissue employed.

Results. Aconitase activity. Enzyme activity was measured in liver homogenates of 26 normal and 36 tularemic rats. The latter were divided into 4 equal groups and aconitase activity was determined 24, 48, 72, and 96 hours following infection. Enzyme activity in livers of normal rats averaged 29.5 ± 1.1† units per mg dry weight of tissue, while activity in homogenates of tularemic livers was reduced within 24 hours after infection to an average of 21 ± 4.1 units. Thereafter, activity dropped more gradually until a level of 19.5 ± 2.1 units was reached at 96 hours following infection (Fig. 1). These changes did not result from inanition since no significant decrease in consumption of food and water by infected rats occurred prior to the fourth post-infection day.

Subsequently, cortisone acetate was administered to infected rats in concentrations of 5 mg per rat per day, starting at time of infection and for 96 hours thereafter. Once again, 36 rats were divided into 4 groups and liver aconitase activity was measured at 24 hour intervals. Enzyme activity dropped slightly after 24 hours but was maintained at 28 ± 2.4 units 48 hours after infection, whereupon it became depressed markedly after 72 hours (Fig. 1). Thus, cortisone

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† Mean ± Standard Error of Mean.

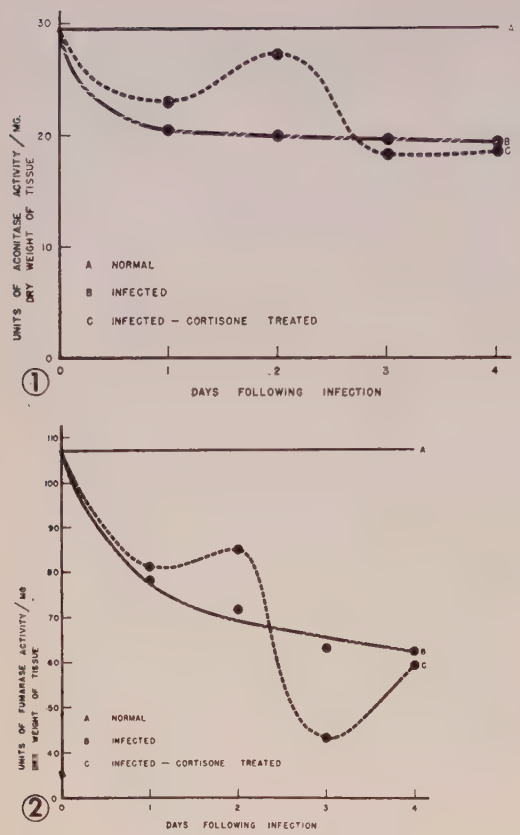

FIG. 1. Comparison of liver aconitase activity in normal and tularemic rats and in tularemic rats given cortisone at a rate of 5 mg/rat/day.

FIG. 2. Comparison of liver fumarase activity in normal and tularemic rats and in tularemic rats given cortisone at a rate of 5 mg/rat/day.

maintained aconitase activity at normal levels 48 hours following infection, compared to 19.7 ± 2.2 units in untreated animals, but subsequently failed to support activity at levels above those observed in infected control rats.

It was determined that cortisone administered as described above was the optimal amount for enhancement of enzyme activity.

Fumarase activity—was measured in liver homogenates of 20 normal animals. Sixteen infected rats were divided into 4 groups and enzyme activity was determined at 24 hour intervals as described above. Fumarase activity in normal rats averaged 108.2 ± 3.3 units per mg dry weight of liver homogenate. The function of this enzyme in infected animals was found to be reduced 24 hours after infection to 78.3 ± 4.1 units while at 48 hours activity was 72.2 ± .1 units. After 72 hours it dropped to a low value of 62.9 ± 4.5 units and remained at that level through the 96 hour period (Fig. 2).

In a fourth experiment, each of 12 infected rats was treated with cortisone acetate as previously described. These also were divided into 4 groups and fumarase activity again determined. Treatment with cortisone maintained fumarase activity at 84.0 ± 9.5 units 48 hours after infection, a level slightly higher than in infected-untreated rats (Fig. 2). Activity after 72 hours was reduced to 43.1 ± 4.0 units, a level much lower than that observed in the infected-untreated rats at the same time interval. However, fumarase function subsequently increased and at 96 hours was comparable to that in the infected control group.

Discussion. The reduction in activity of liver aconitase and fumarase during the course of infection suggests that depression of energy metabolism is responsible for some of the pathology observed in tularemia. Also, our observation that the activity of these enzymes can be supported temporarily at nearly normal levels by treatment with cortisone suggests adrenal involvment, since the adrenals are known to exert control over carbohydrate metabolism.

Evidence for this concept is supplied by Pinchot and his co-workers(3) who observed that adrenal exhaustion accompanied by a pronounced drop in body temperature occurred several hours prior to death in tularemic rats. Treatment with adrenal cortical hormone did not relieve adrenal exhaustion or reduce mortality but the animals were better able to maintain body temperature and seemed more active than the controls.

From both of these studies, it appears that adrenal damage was too extensive to be compensated adequately by therapeutic use of cortisone. However, it is well recognized that *P. tularensis* exhibits a high degree of invasiveness resulting in the parasitism of nearly all body tissues. Thus, disturbances in other vital metabolic functions may occur as a result of infection which also contribute

to the severe symptoms noted in tularemia.

Nevertheless, impairment of normal energy yielding reactions seems to be a critical factor influencing the course of infection. An analogy to this is provided by Gilfillan *et al.* (4,5) who studied the adverse influence of *Salmonella pullorum* on liver enzymes of the tricarboxylic acid cycle in baby chicks. They, likewise, concluded that many of the symptoms of the infection were referable to disturbances in this particular metabolic pathway.

Although these comparisons are made between 2 unrelated hosts and 2 unrelated bacterial species, there appears to be an underlying similarity in fundamental pathology in each instance. Gilfillan and his collaborators(5) have suggested the possible role of endotoxins in producing the abnormalities they observed. The work of Berry *et al.*(6) substantiates this concept. They demonstrated that mice poisoned with endotoxin of *Salmonella typhimurium* were depleted of nearly all carbohydrate. However, poisoned mice given a protective dose of cortisone maintained carbohydrate levels 2 to 3 times as great as that of the poisoned controls. Quite possibly, similar effects may be produced in rats by action of an endotoxin of *P. tularensis* which has been demonstrated and characterized by Ormsbee and Larsen(7) and which appears to be related to those of other gram-negative bacteria.

An evaluation of the effects of specific endotoxins on host metabolism would be most desirable in studies of tularemic infection.

Summary. Aconitase and fumarase activity in liver tissue of tularemic rats was demonstrated to be reduced by more than 33% below normal values at height of infection. Administration of cortisone in a concentration of 5 mg per rat per day during infection supported aconitase activity at nearly normal levels for 48 hours but failed to do so beyond that time. Liver fumarase activity of rats treated with cortisone was maintained at only slightly higher levels than observed for the controls. The data presented suggest extensive adrenal involvement in tularemic rats which can be alleviated only partially and temporarily by daily administration of cortisone.

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Connective Tissue V. Comparison of Synthesis and Turnover of Collagen and Elastin in Tissues of Rat at Several Ages.* (26329)

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Previous work from this laboratory has shown that collagen of several tissues of rat increased with age while elastin content remained relatively constant(1). Since there are variations in rates of synthesis and turnover of collagen in several tissues of growing rats(2), it becomes important to study the synthesis and turnover of collagen and elastin in tissues of rats as a function of age.

Methods. Female rats, Wistar strain, 5 weeks, 8 months and 2 years of age, respectively, were injected intraperitoneally with saline containing uniformly labeled C^{14}-lysine (7.8 μc per 100 g body weight). Groups of 3-6 rats were sacrificed at 1, 3, 10, 20, 30 and 40 days, respectively, after injection.

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TABLE I. Synthesis and Turnover of Proteins of Aorta of Female Rat.

(Figures are expressed as relative specific activity ± stand. dev. Assuming maximum specific activity as 100, the specific activity of the remaining days is expressed as % of maximum specific activity.)

Proteins	Age	No.	1 day	3 day	10 day	20 day	30 day	40 day
Nonscleroprotein	5 wk	1–3*	100 ± 39 (222)†	73 ± 10	43 ± 5		20	10
	8 mo	4–5	100 ± 18 (154)	89 ± 4	53 ± 5	30 ± 5	29 ± 1	22 ± 3
	2 yr	4	100 ± 49 (259)	56 ± 8	40 ± 7	27 ± 5	22 ± 4	18 ± 3
Soluble collagen	5 wk	1–3*	100 ± 15 (140)	61 ± 25	40 ± 11		17	9
	8 mo	4–5	91 ± 9	100 ± 17 (77)	24 ± 6	31 ± 11	21 ± 1	24 ± 3
	2 yr	4	100 ± 13 (198)	43 ± 3	27 ± 3	24 ± 6	13 ± 4	16 ± 1
Insol. "	5 wk	1–3*	71 ± 12	93 ± 30	100 ± 12 (41)		78	54
	8 mo	4–5	100 ± 61 (18)	78 ± 44	57 ± 6	57 ± 6	57 ± 11	57 ± 17
	2 yr	4	64 ± 21	100 ± 7 (14)	50 ± 7	29 ± 21	32 ± 7	43 ± 28
Elastin	5 wk	1–3*	57 ± 12	70 ± 27	67 ± 9		100 (33)	70
	8 mo	4–5	75 ± 10	25 ± 15	100 ± 50 (4)	100 ± 25	50 ± 35	100 ± 75
	2 yr	4	25 ± 0	38 ± 50	30 ± 50	25 ± 50	50 ± 50	100 ± 50 (2)

* Combined sample from 2-3 rats.
† Figures in parentheses are actual SA (cpm/mg of protein) observed.

TABLE II. Synthesis and Turnover of Proteins of Uterus of Female Rat.

(Figures are expressed as relative specific activity ± stand. dev. Assuming maximum specific activity as 100, the specific activity of the remaining days is expressed as % of maximum specific activity.)

Proteins	Age	No.	1 day	3 day	10 day	20 day	30 day	40 day
Nonscleroprotein	5 wk	1–3*	100 ± 30 (328)†	82 ± 30	31 ± 2		6	4 ± 1
	8 mo	4–5	100 ± 6 (386)	86 ± 30	27 ± 6	10 ± 2	7 ± 2	5 ± 1
	2 yr	4	100 ± 9 (560)	66 ± 1	25 ± 0	10 ± 1	7 ± 1	5 ± 1
Soluble collagen	5 wk	1–3*	100 ± 45 (220)	99 ± 34	34 ± 3		11 ± 5	4 ± 2
	8 mo	4–5	100 ± 8 (262)	94 ± 20	24 ± 9	12 ± 3	8 ± 0	6 ± 0
	2 yr	4	100 ± 18 (330)	64 ± 13	26 ± 5	15 ± 9	9 ± 1	8 ± 2
Insol. "	5 wk	1–3*	56 ± 22	100 ± 67 (101)	88 ± 21		30	19 ± 4
	8 mo	4–5	34 ± 8	100 ± 52 (77)	33 ± 13	38 ± 14	35 ± 4	21 ± 6
	2 yr	4	43 ± 28	100 ± 46 (35)	86 ± 28	100 ± 32	76 ± 20	40 ±14

* † See legend, Table I.

TABLE III. Synthesis and Turnover of Proteins of Tendon of Female Rat.
(Figures are expressed as relative specific activity ± stand. dev. Assuming maximum specific activity as 100, the specific activity of the remaining days is expressed as % of maximum specific activity.)

Proteins	Age	No.	1 day	3 day	10 day	20 day	30 day	40 day
Nonscleroprotein	5 wk	3–5	69 ± 9	100 ± 10 (163)†	56 ± 14		20 ± 2	13 ± 2
	8 mo	4–5	100 ± 14 (135)	95 ± 8	52 ± 7	27 ± 7	21 ± 2	18 ± 6
	2 yr	4	100 ± 46 (347)	46 ± 8	22 ± 2	19 ± 1	13 ± 9	9 ± 1
Soluble collagen	5 wk	3–5	48 ± 4	100 ± 18 (77)	66 ± 18		34 ± 4	20 ± 5
	8 mo	4–5	18 ± 8	92 ± 38	100 ± 39 (13)	77 ± 15	54 ± 15	77 ± 30
	2 yr	4	90 ± 30	100 ± 60 (10)	70 ± 80	50 ± 50	30 ± 20	40 ± 10
Insol. "	5 wk	3–5	17 ± 6	58 ± 14	75 ± 17		100 ± 6 (36)	44 ± 6
	8 mo	4–5	0	40 ± 40	40 ± 40	100 ± 40 (2.5)	40 ± 40	100
	2 yr	4	0	0	0	0	0	0

† Figures in parentheses are actual SA (cpm/mg of protein) observed.

Tissues from aorta, tendon, uterus and skin were fractionated into nonscleroprotein (NSP), soluble collagen (SC), insoluble collagen (IC) and elastin (E) as described before(2). Methods employed in chemical determinations and radioisotopic procedures have been previously detailed(2).

Results. Rates of synthesis and turnover of protein moieties in aorta, uterus, tendon and skin at different ages are compared in Tables I, II, III and IV.

With age, there was no difference in the synthesis and turnover of NSP in uterus, tendon and skin, while the turnover rate of NSP in aorta was slower at 8 months and 2 years, respectively, than at 5 weeks.

In all tissues studies, the turnover rates of SC were slower at 8 months and 2 years, respectively, than at 5 weeks. The maximum specific activity (SA) of SC in tendon was significantly higher in rats 5 weeks old than in those 8 months and 2 years old, respectively.

In aorta, SA of IC was significantly higher at 5 weeks than at 8 months and 2 years. At the 2 latter ages, it decreased to approximately half in 10 days with little decay thereafter, and at 5 weeks reached a similar level at the end of 40 days. In uterus no apparent difference occurred in the maximum SA of IC at any age. However, the decay of IC was slowest at 2 years of age.

In tendon, the SA of IC was highest at 5 weeks of age. Synthesis was barely detectable at 8 months and not measurable in the 2 years old animal. The decay of IC in tendon was measurable only at 5 weeks.

In skin, the SA of IC was higher at 5 weeks of age than at 8 months and the SA of IC was higher at 8 months than at 2 years. Appreciable decay of IC in skin was observed only at 5 weeks.

The SA of elastin in aorta was significantly higher at 5 weeks than at 8 months or 2 years. A detectable decay of elastin in the aorta occurred only in 5-week-old rats.

Discussion. With the exception of the uterus, IC and elastin were synthesized at significantly higher rates at 5 weeks of age than at 8 months or 2 years, and the IC of tendon and skin showed a higher SA at 8 months than at 2 years.

These data indicated that the concentration of newly formed collagen in the several tissues studied and of newly formed elastin in the aorta were highest at an early age. The turnover rates of collagen in tendon, skin and aorta are relatively slow; these results

TABLE IV. Synthesis and Turnover of Proteins of Skin of Female Rat.

(Figures are expressed as relative specific activity ± stand. dev. Assuming maximum specific activity as 100, the specific activity of the remaining days is expressed as % of maximum specific activity.)

Proteins	Age	No.	1 day	3 day	10 day	20 day	30 day	40 day
Nonscleroprotein	5 wk	3–6	100 ± 17 (221)†	87 ± 8	41 ± 9		10 ± 1	7 ± 1
	8 mo	4–5	100 ± 16 (220)	87 ± 15	30 ± 11	19 ± 4	13 ± 1	10 ± 2
	2 yr	4	100 ± 32 (334)	61 ± 10	34 ± 6	19 ± 2	9 ± 3	8 ± 3
Soluble collagen	5 wk	3–6	100 ± 8 (160)	80 ± 17	40 ± 11		10 ± 1	9 ± 2
	8 mo	4–5	100 ± 26 (73)	96 ± 22	43 ± 11	34 ± 6	18 ± 3	16 ± 3
	2 yr	4	100 ± 38 (102)	47 ± 12	26 ± 5	20 ± 5	17 ± 4	15 ± 5
Insol. "	5 wk	3–6	51 ± 10	100 ± 26 (61)	84 ± 16		41 ± 11	41 ± 3
	8 mo	4–5	42 ± 17	58 ± 25	67 ± 33	100 ± 25 (12)	67 ± 8	83 ±21
	2 yr	4	25 ± 25	63 ± 37	43 ± 25	95 ± 25	100 ± 75 (4)	50 ±50

† See legend, Table III.

are in accord with Neuberger's work on the collagen of rat tendon(3). The highest SA for collagen was found in uterus, for which decay values were also highest and were observed even in old age. In aorta, in the older age groups, there was a turnover of collagen during the first 10 days following administration of C^{14}-lysine, after which no further loss of material was observed. This may indicate at least 2 distinct insoluble collagen components. Such nonhomogeneity of collagen has been reported for various soluble collagens(4,5,6,7,8). The elastin of aorta did not decay in activity in the older age groups, a finding which agrees well with the data of Slack(9).

Summary. 1. Synthesis and turnover of collagen and elastin was studied in rats of 5 weeks, 8 months and 2 years of age, respectively, by observing incorporation and removal of uniformly labeled C^{14}-lysine. 2. Synthesis of collagen and elastin was more rapid in the tissues of young rats (5 weeks) than in those of older animals. In tendon, no detectable synthesis of IC was found at 2 years of age. 3. The uterus was the only organ examined, which showed appreciable synthesis and turnover of collagen in older animals. 4. The turnover rate of collagen in relation to other proteins was low in all animals even in those 5 weeks old. No turnover was observed in the insoluble collagen of tendon and skin at 8 months and 2 years of age, respectively.

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Incorporation of Infused Palmitic-1-C^{14} and Linoleic-1-C^{14} into Plasma Lipid Fractions.* (26330)

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In recent years, there has been increasing recognition of the major contribution of free fatty acids (FFA) to the energy needs of nearly all body tissues. Several studies have clearly shown that infused FFA disappear rapidly from the general circulation, and are oxidized, in part, directly by tissues. The metabolic fate of that portion which does not undergo direct oxidation has been the subject of many studies. It has been shown that a considerable portion of administered palmitic acid-1-C^{14} is incorporated by the liver into triglycerides and, to a lesser degree, into phospholipids(1,2). These studies have involved primarily an analysis of liver lipids rather than plasma lipids. Laurell(3), on the other hand, reported the disappearance and recycling, as esterified fatty acid, of injected palmitic acid-1-C^{14} and found that peak incorporation into plasma triglycerides occurred at about 40 minutes. Fredrickson and Gordon(4) infused palmitic-1-C^{14}, linoleic-1-C^{14}, and oleic-1-C^{14} acids into humans and found initial disappearance rates to be similar. Although their flux data indicate that basic differences do exist between the metabolism of linoleic and palmitic acids, they did not find significant differences in incorporation of these acids into the non-phospholipid-containing neutral lipid fraction. In a study in which acetate-1-C^{14} was administered orally to humans, Lipsky(5) was able to follow the metabolic fate of the fatty acids synthesized. He found that palmitic-C^{14} acid incorporation into triglycerides, phospholipids, and sterol esters occurred maximally at 2, 24, and 48 hours respectively. Although peak incorporation of linoleic-C^{14} acid into triglycerides occurred at 2 hours also, the activity of sterol esters and phospholipids derived from this fatty acid was too low to permit conclusions regarding its ultimate fate in plasma lipid fractions. The present study was undertaken to investigate possible differences in incorporation of a typical saturated (palmitic) and a typical unsaturated (linoleic) fatty acid into various plasma lipid fractions.

Materials and methods. Mongrel dogs, weighing 10-20 kg, and fasted overnight were used for this study. Forty microcuries (0.7 mg) of palmitic-1-C^{14} or linoleic-1-C^{14} bound to dog plasma were infused intravenously into three or more dogs over a 2-3 minute period. Blood samples were taken at varying intervals up to 72 hours after infusion. The animals were fed after the 6-hour sample, and all subsequent samples were taken at least 3 hours after a meal. The blood was placed in chilled, citrated tubes in an ice bath, and promptly centrifuged at 4°C in the cold room. A 1-cc portion of plasma was extracted by the method of Freeman(6). Extracts were evaporated, taken up in hexane, and placed on silicic acid columns. The various lipid fractions were eluted using the solvent systems described by Dittmar(2). The fraction containing triglycerides and FFA was extracted 3 times with water-isopropyl alcohol (1:2) made basic (pH 9.0) with dilute NaOH. Subsequent acidification of the aqueous extract with 0.1 N HCl and extraction with 3 volumes of hexane removed the FFA. All eluted fractions were then evaporated to dryness, and counted in toluene-PPO in a liquid scintillation counter.

Results. Both the linoleic-C^{14} and the palmitic-C^{14} disappeared from the plasma very rapidly—more than 90% in one hour. The palmitic-C^{14} had largely disappeared after 5 hours and the linoleic-C^{14} slightly more slowly. Each were incorporated into plasma triglyceride at a comparable rate and continued at similar concentrations over 72

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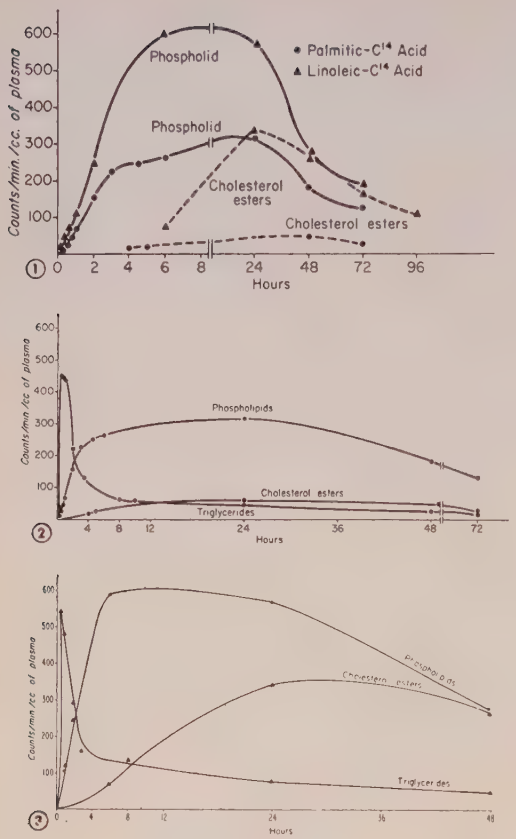

FIG. 1. Each labeled fatty acid is incorporated much more rapidly into phospholipid than into cholesterol esters. More linoleic-C^{14} than palmitic-C^{14} is incorporated into both lipids.

FIG. 2. Palmitic-C^{14} is incorporated very rapidly into triglycerides, more slowly into phospholipids, and very slowly into cholesterol esters.

FIG. 3. Linoleic-C^{14} is incorporated rapidly into triglyceride, more slowly into phospholipids, and very slowly into cholesterol esters. Comparison with Fig. 2 demonstrates that linoleic-C^{14} is incorporated into phospholipid and cholesterol esters faster than palmitic-C^{14}.

hours. The highest labeled triglyceride concentrations were attained between one-half and one hour.

Much greater concentrations of labeled plasma phospholipid and cholesterol esters were obtained after administration of linoleic-C^{14} than after palmitic-C^{14} (Fig. 1). In each instance the incorporation of the labeled material occurred most rapidly in triglyceride, then phospholipid and then cholesterol esters (Fig. 1, 2, 3). Incorporation into cholesterol esters is very slow, no significant activity being found until after 2 or 3 hours; in each instance maximum activity was reached in about 24 hours. On the other hand, significant activity was found in the phospholipid fraction within 10 minutes of the infusion of the FFA, and peak incorporation occurred at about 6 hours.

Discussion. The major difference found in this study of incorporation of palmitic-C^{14} and linoleic-C^{14} acids into various plasma lipid fractions consisted of a quantitatively greater incorporation of linoleic-C^{14} acid into sterol esters and phospholipids. Lipsky(5) has shown that linoleic-C^{14} acid synthesized after oral administration of acetate-1-C^{14} to humans is preferentially incorporated into sterol esters and the phospholipid fraction of serum lipids, while palmitic-C^{14} acid appears to be primarily esterified with glycerol. It was also noted in his study that peak incorporation of palmitate-C^{14} into triglycerides, phospholipids, and sterol esters occurred at 2, 24, and 48 hours respectively. The time course of events found in our experiments suggests that earlier peak activities (45 minutes, 6 hours, 24 hours) are found when the FFA (rather than acetate-1-C^{14}) are used as the starting material and are given intravenously.

Amount of fatty acid incorporation in various lipids may differ with route of administration, species, and other factors. For example, Hanahan and Blomstrand(7), administering FFA orally to rats, found that 10 times as much radioactivity was incorporated in lecithins after administration of palmitic-1-C^{14} acid as after administration of oleic-1-C^{14} acid.

Our observation that linoleic-C^{14} acid remains longer as a FFA following intravenous administration than palmitic-C^{14} acid has been described by Fredrickson and Gordon (4), whose data indicate that linoleic-C^{14} acid is oxidized less rapidly and recycles through the plasma compartment much more than either palmitic-C^{14} or oleic-C^{14} acids.

It is evident that more rapid oxidation of palmitic-C^{14} acid would result in diminished amounts available for synthesis into other lipid fractions, and hence could account for some quantitative differences observed in

subsequent synthetic processes. Such a mechanism cannot account for differences in slopes of the curves, however, and therefore certain preferential esterifying mechanisms for each of the fatty acids must be operative.

Summary. Palmitic-1-C^{14} and linoleic-1-C^{14} acids were infused into fasted dogs, and fractionation of the serum lipids carried out at time intervals up to 72 hours. Although many similarities were found in the metabolism of these 2 fatty acids, it was observed that linoleic-C^{14} acid appeared to 1) persist longer in the plasma as the FFA and 2) to be incorporated preferentially into cholesterol esters and phospholipids.

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Hypoxic Alteration of Righting Ability and Pain Threshold in Two Mammalian Species.* (26331)

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Although much experimental work has been done in man and animals on pain(1), there are only limited studies on the effects of hypoxia on thermal pain perception(2). Besides studying hypoxic effects on pain thresholds, we were also interested in using the response to pain along with tests of righting ability as indices of body function during hypoxic episodes.

Mammalian species that hibernate survive more severe hypoxia than do non-hibernating species(3,4). Two possible means by which hypoxic tolerance could be greater in one species than another are: a) the more tolerant mammal possesses greater capacity to maintain high functional levels by maintaining tissue oxygenation through greater utilization of physiological regulatory mechanisms or biochemical energy reserves. b) The more tolerant mammal by decreasing metabolism and function, reduces the oxygen requirement to parallel the reduced oxygen supply.

In the present study, an attempt was made to evaluate the above alternatives as to their role in species differences of hypoxic tolerance. Degree of hypoxic alteration of function as shown in pain threshold and righting ability changes was compared for one hibernating species and one non-hibernating species.

Methods. Experimentally naive, Harlan strain white rats (*Rattus norvegicus*) of 200 to 300 g body weight were the non-hibernators used. Thirteen lined ground squirrels (*Citellus tridecimlineatus*) of 150 to 250 g body weight were the hibernators used.

Pain threshold was determined by using a modification of the method described by D'Amour and Smith(5). The tails of the ground squirrels were shaved prior to the experiment. Animals of both species were restrained either in cylinders or taped to boards. A small hood was placed over the head of the animal through which 1 to 2 liters per minute of air, or an air mixture deficient in oxygen were pumped. The tail was then placed in a trough containing a resistance wire heater. The heater was turned on and the temperature increased at approxi-

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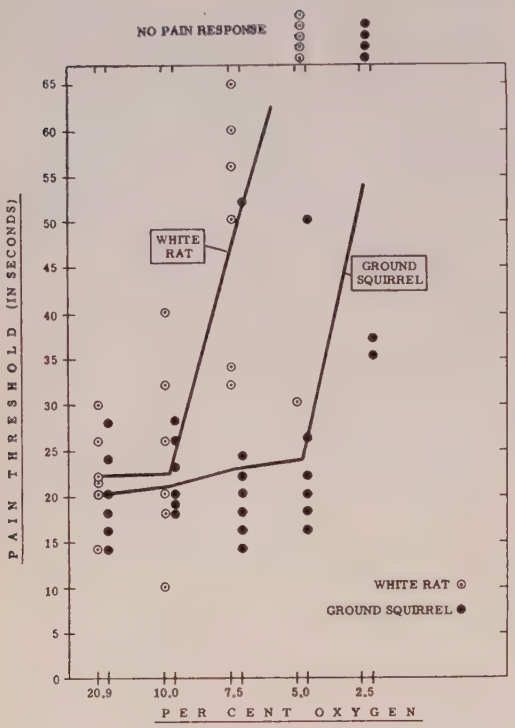

FIG. 1. Effects of varying inspired oxygen percentage on pain threshold (tail flick response time) in white rats and ground squirrels. Each point represents one determination. Curves are drawn through the mean at each oxygen percentage.

mately 4°C per second. The reflex reaction time required for the first definite tail twitch was taken as the pain threshold. In tests where no response was obtained, the tail was removed at the first indication of burning. Hypoxic tests were done after 5 minutes of breathing a low oxygen mixture. The method gave highly reproducible results for both species.

The degree of hypoxia necessary to abolish the righting reflex was determined by placing the animal in a 3 liter jar which could be rotated while air was being pumped out. In one series, the rate of air removal was equivalent to an altitude ascent of 2,000 feet per minute. The end point was taken at the first sign of inability of the animal to right itself in the rotating jar and barometric pressure was then measured.

In a second series, the animals were decompressed to 150 mm Hg in 9 seconds. Time required for loss of righting ability was then measured.

Results. The mean normal pain threshold in our apparatus was 22 seconds in the rat and 19 seconds in the ground squirrel. Little alteration in pain threshold occurred in the rat until the oxygen percentage was below 10 (Fig. 1) Five per cent oxygen abolished pain sensitivity in 5 out of 6 rats. All of the rats survived the brief (about 7 minutes) hypoxic period of 5% oxygen breathing.

The ground squirrels showed no depression of pain sensitivity with air mixtures containing 5% oxygen. Of the 6 animals at 2.5%, 2 still showed a response after 5 minutes. One animal did not survive the test, but 5 animals recovered completely within a few minutes after this hypoxic episode.

Righting ability was less susceptible to hypoxia in the rat for both series of tests (Table I). The rat appeared to fight the hypoxia and struggled to maintain the upright position. At the end of the hypoxic episode, the rat required several minutes to regain consciousness and complete motor control. The ground squirrels on the other hand, made little effort to fight the jar rotation even in the first few seconds of deep hypoxia but seemed content in the supine position. Recovery in the ground squirrel was much faster than that of the rat following hypoxia.

Discussion. Our results from animals are in agreement with those of Stokes *et al.*(2) who found that in men breathing 10% oxygen there was no change in thermal pain perception. These authors could not use with

TABLE I. Barometric Pressure at Which Righting Was Lost.

Animal	No. of obs.	Pressure (mm Hg)	Equivalent oxygen (%)
Rat	6	154.0 ± 9.0*	4.3
Ground squirrel	6	183.0 ±10.0	5.1

Time righting ability remained after 9-second ascent to 38,000 feet (150 mm Hg or equivalent to 4.2% oxygen)

Animal	No. of obs.	Time (sec.)
Rat	9	52.0 ± 11.0*
Ground squirrel	9	8.1 ± 1.5

* Mean ± S.D.

humans the severe hypoxia that we used to alter pain threshold in animals.

In comparison of the 2 species, ground squirrels maintained pain sensitivity with more severe hypoxia than could the rat, but the rat's righting ability was much more susceptible. The response to pain as we measured it may be a spinal reflex(1) while the righting response utilizes higher centers. This indicates that the ground squirrel supported some functions, perhaps at the expense of others, during oxygen deficiency. The greater hypoxic survival of the ground squirrel could be due to partial functional depression which conserves energy for maintenance of more vital functions. Which functions are maintained and how they are maintained, and which are depressed are avenues of future research.

Summary. The response to thermal pain in the rat was decreased by breathing 7.5% oxygen and abolished with 5% oxygen. The 13 lined ground squirrel had no alteration of response with 5% oxygen but did with 2.5%. However, the righting reflex of the ground squirrel was much more susceptible to hypoxia than that of the rat. Loss of righting ability occurred at a barometric pressure of 183 mm Hg for the ground squirrel and 154 mm Hg for the rat in simulated altitude ascent in a chamber.

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Threshold and Pattern of Electroshock Seizures in Ataxic Manganese-Deficient Rats.* (26332)

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Offspring of manganese-deficient females exhibit a congenital ataxia characterized by incoordination, loss of equilibrium, retraction of the head, and tremor. This abnormal condition has been shown to result from an irreversible defect or defects occurring between the 14th and 18th days of gestation in rats (1). Recent studies, both physiological and morphological, have revealed abnormalities of the inner ear in manganese-deficient young; these studies indicate that impaired vestibular function may be involved in the etiology of the ataxic syndrome(2,3,4). However, certain manifestations of the ataxia, such as tremor, could not be attributed to loss of vestibular function. It was therefore of interest to examine another aspect of nervous system function.

The present communication describes experiments investigating brain excitability, as measured by response to electroshock, in offspring of normal and manganese-deficient rats.

Materials and methods. Animals and diets. Ataxic, manganese-deficient offspring were produced by procedures similar to those previously reported(3). Briefly, female rats of the Sprague-Dawley strain were maintained from time of weaning on a fortified milk diet deficient in manganese. Control groups were treated in the same way, except that manganese was added to the diet.‡ At maturity the animals were mated with normal males.

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† U.S.P.H.S. Pre-doctoral Fellow.

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Deficient females continued to receive the manganese-deficient milk ration during pregnancy and lactation, except for 24 hours on the 16th day of gestation,§ when they were given the manganese-supplemented ration used for control animals.‖ After weaning at 28 days of age, the young were maintained on diets received by their mothers (either manganese-deficient or manganese-supplemented). All of the manganese-deficient rats used in this study showed marked ataxia.

Electroshock technics. The apparatus and methods for producing electroshock seizures in rats have been described in detail by Woodbury and Davenport(5), and are at present used routinely in several laboratories. These methods are: 1. *minimal electroshock seizure threshold (EST) test,* as a measure of brain excitability; 2. *maximal electroshock seizure (MES) pattern test,* as a measure of spread of excitation; and 3. *return of the righting reflex (RRR) test,* as a measure of brain recovery processes.

The EST is defined as the minimal amount of alternating-current in milliamperes (ma), delivered for 0.2 seconds through corneal electrodes, which will just elicit barely detectable clonic convulsions of ears, jaws, and forepaws without loss of equilibrium and lasting only a few seconds. The MES is produced by delivering 150 ma for 0.2 seconds through corneal electrodes. With this supramaximal stimulus, normal rats show typical maximal (tonic-clonic) convulsions. Total duration of the seizure, duration of the tonic phase with its flexor and extensor components and duration of the clonus are determined for each animal. The RRR test measures duration of the depression period elapsing between the end of MES and the spontaneous rise of the animal.

In the present experiments, EST was determined 2 or 3 times a week for 2 weeks or until threshold stabilization occurred. At least one day of rest was allowed between tests. Three days after the last EST test, the MES pattern and the RRR were determined. Body weight was measured at beginning and end of the electroshock testing period.

Results. The effects of manganese deficiency on EST in rats are summarized in Table I. The EST was lower in the deficient rats than in the corresponding normal controls of the same age; the difference in EST was statistically significant.

Body weight of the manganese-deficient rats was smaller than that of the controls. This is in agreement with previous experiments(6) in which a slower weight gain was observed in deficient rats as compared with their controls. On the other hand, the electroshock testing did not interfere with the growth of either control or deficient rats as judged by the increase in body weight with time during the experimental period.

The effects of manganese deficiency on MES and RRR in rats are also presented in Table I. A statistically significant decrease was observed in duration of the flexor tonic phase in the manganese-deficient rats as compared with the manganese-supplemented controls. The other parameters of the maximal convulsions were similar in the two groups of animals. The RRR was longer in the deficient rats but the difference with the controls was not statistically significant.

Discussion. The data presented indicate that ataxic offspring of manganese-deficient rats, maintained on a deficient diet, show a marked increase in brain excitability as measured by an EST significantly lower than in normal rats. It may be noted that the manganese-deficient rats were smaller in size than the corresponding controls of the same age. In view of the positive correlation between body weight and EST described elsewhere (7) it might be postulated that the lower EST in the manganese-deficient animals is related to the smaller body weight. However, both phenomena, greater excitability and smaller body size, may be concomitant and yet independent expressions of the same metabolic factor, in this case, manganese deficiency.

§ The day of finding sperm in the vaginal smear is considered day zero.

‖ Supplementation for one day during gestation was used as a means of increasing survival of the young. Data on the effects of this procedure on survival will be presented later.

TABLE I. Minimal Electroshock Seizure Threshold (EST), Maximal Electroshock Seizure (MES) Pattern and Postictal Depression in Normal and Manganese-Deficient Rats.

Description	No. of rats	Age, days	Body wt (g) Initial	Body wt (g) Final	EST,* mAmp	MES† pattern, duration of each phase (sec.) Tonic flexion	Tonic extension	Total tonus	Clonus	Total seizure	Postictal depression, min.‡
Normal controls	17	80	181.2§ ±9.4	184.5 ±9.2	21.09 ±.08	3.83 ±.35	11.21 ±.52	15.05 ±.41	17.56 ±1.42	32.61 ±.76	2.06 ±.14
Manganese-deficient	10	80	137.4 ±7.1	143.8 ±7.1	18.60 ±.36	1.67 ±.31	12.90 ±1.15	14.98 ±.47	18.06 ±3.18	33.06 ±3.23	3.11 ±1.42
P‖ (control *vs* manganese deficient rats)					<.01	<.01	<.001	<.01			

* EST is defined as minimal amount of alternating current delivered for 0.2 sec. through corneal electrodes which will elicit barely detectable clonic convulsions of ears, jaws and forepaws without loss of equilibrium and lasting only a few seconds.

† MES were produced by delivering 150 milliamperes of a.c. for 0.2 sec. through corneal electrodes.

‡ Postictal depression after a maximal seizure was the time required to recover righting reflex.

§ Mean ± stand. error.

‖ P values based on t-tests for significance of difference between means.

The increased brain excitability in the manganese-deficient rats is also evidenced by changes in the MES pattern. Toman *et al.* (8) showed that the response to a supramaximal stimulus, such as the one used in producing maximal seizures, is independent of current density and of the passive influence of body mass. In the present study, a shorter duration of the tonic flexion was observed during the MES in the manganese-deficient rats. Duration of the tonic flexion is considered to be proportional to intensity of the inhibitory state which must be overcome before the maximal spread of excitation occurs. Maximal spread of excitation is indicated by appearance of the tonic extension. Thus, the shorter the flexion, the shorter the period of inhibition and the greater the excitability. The fact that a higher degree of convulsability could be demonstrated in the manganese-deficient animals with both tests used, whether correlated (EST) or independent (MES) from body mass, adds further support to the conclusion that brain excitability is increased by manganese deficiency.

These results are consistent with the gross behavior of the deficient animals: they were hyperactive and appeared to be hypersensitive to stimuli.

Previous researches seeking to determine the origin of the congenital ataxia of manganese-deficient animals have failed to reveal any histological lesions in the central nervous system(9,10,11,1). The present study may indicate that the brain is involved in the ataxic syndrome of manganese-deficient offspring. This work does not show, however, whether the increased brain excitability observed in this study is the result of an irreversible congenital defect, as is known to be the case in regard to ataxia(1), or whether it results from a deficiency of circulating manganese as such.

Summary. Electroshock convulsions were produced in ataxic manganese-deficient rats. The threshold for minimal seizures was significantly lower in the manganese-deficient than in the control animals. During a maximal seizure, duration of the tonic flexor phase was markedly shorter in the deficient than in control rats; duration of the other phases and of postictal depression were similar in both groups. The data are interpreted to mean that brain excitability and convulsability are increased in ataxic manganese-deficient rats; these data may indicate that the brain is involved in the ataxic syndrome characteristic of offspring of manganese-deficient animals.

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Facilitation of Pituitary-Induced Frog Ovulation by Progesterone in Early Fall.* (26333)

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Since the original investigations of Wolf (1) on *Rana pipiens* and Houssay *et al.*(2) on *Bufo arenarum,* induction of ovulation in anurans by homoplastic pituitary injections or implantations has become a common laboratory procedure. Freshly-ovulated eggs for fertilization studies and embryological material may be obtained at any time during the academic year, but minimal effective dosages of pituitary glands vary widely with the season(3). Nearly complete ovulation in March or April may be induced in the average frog 24 hours following injection of 1 or 2 frog anterior pituitary lobes, but 3 to 4 lobes are usually required in January and February. From mid-September often into December, significant ovulation in a single female necessitates slaughter of 6 to 8 other females, only for their pituitary glands; and 48-56 hours must ordinarily elapse following injection before any appreciable response is obtained. Recent success with steroids in inducing ovulation *in vitro* in *Xenopus laevis* (4) and in *Rana pipiens*(5,6) has quite naturally led into a study of possible use of steroid hormones as substitutes for part or all of the large pituitary dosages required for ovulation *in vivo* in the fall.

Methods. Frogs for these studies were obtained from both Vermont and Wisconsin. Suppliers were instructed not to ship animals which had been kept in a warm room. Frogs were stored upon arrvial in large aquaria in a refrigerator (5°C) with a little water, changed frequently. Pituitary glands were dissected from freshly-killed animals, ground in a small mortar, and injected (#27 needle) in a small volume of Ringer's solution into the dorsal lymph sac. Progesterone or other steroid, as aqueous suspension or dissolved in oil, was administered similarly. Following injection, each animal was kept at room temperature in a large finger bowl containing about a half inch of water and covered to prevent escape but loosely enough to admit air. Evidence of ovulation was often indicated by ability to force ova from the ovisacs, but percentages of ovulation were determined by laparotomy.

Results. Very satisfactory responses (Table I) were obtained when frogs were injected with a single pituitary gland in combination with from 0.5 to 5.0 mg progesterone. Ova could be stripped in profusion from recipients within 12-16 hours following injection. The fact that these ova were readily fertilizable and underwent normal development into swimming larvae indicates that ovulation so obtained is entirely normal. At the time of laparotomy (24 hours following injection), the coeloms of these animals were still crowded with freshly-ovulated eggs, signifying that abundant ovulation was still in process.

Small amounts of ovulation were also obtainable by injecting progesterone alone

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TABLE I. Ovulation Induced in *Rana pipiens* in October by Injection of One Triturated Frog Pituitary Gland in Combination with Progesterone.

Progesterone dosage, mg	No. frogs	No. frogs responding	Avg % ovulation*
0	6	0	0
.1	4	1	2
.5	"	4	40
1.0	"	"	60
2.0	"	"	75
5.0	"	"	85

* Determined by laparotomy 24 hr following inj.

TABLE II. Ovulation Induced in October by Injection of Progesterone Alone.

No. progesterone injections	Total progesterone admin., mg	No. frogs	No. frogs responding	Avg % ovulation*
2	1	2	0	0
2	2	2	1	1
1	5	3	3	5
2	10	4	4	10
2	20	2	2	1
2	25	2	0	0
1	25	2	Died	—

* Determined by laparotomy 48 hr after first inj.

(Table II), but this steroid obviously is not an effective substitute for pituitary factors, at least in October. It is interesting that optimal response, even though it was only about 10%, followed a total dosage of 10 mg, and that larger amounts were apparently inhibitory. A single dosage of 25 mg progesterone was lethal within 1 hour. Those animals giving positive responses to progesterone alone had a tendency to force ova from the ovisacs spontaneously, whereupon the jelly coat begins to swell immediately; such eggs are of no use as potential embryological material since they cannot be fertilized. Testosterone in oil, in total dosages of 5 and 10 mg, also induced 5-10% ovulation (4 frogs), but larger amounts seemed to be even more toxic than progesterone and did not induce ovulation.

Discussion. Progesterone's ability to augment the ovulatory effect of pituitary factors is evident in the foregoing data. It is particularly interesting that progesterone markedly reduced both the dosage of pituitary hormone (by a factor of 6 or more) and time required for maximal response (by a factor of 2 or 3). Although these experiments were performed in October, there is every reason to suppose that similar results could be obtained in September or even August. Since pituitary-induced ovulation in October from frog ovarian lobes *in vitro* was also found to be augmented by progesterone (by a factor of 3), it is assumed that the effects noted *in vivo* are direct and not mediated by the recipient's own pituitary gland. The possibility remains, however, that progesterone could facilitate release of pituitary gonadotropin as has been observed in rabbits(7) and fowl(8). A repeat of the experiments in Table I, substituting testosterone in oil for progesterone, gave positive results (40% ovulation) only at the 5 mg steroid level. Substitution of an aqueous suspension of cortisone† for testosterone in another repeat gave only 1-2% ovulation at both 2 and 5 mg steroid dosages. It is notable, though, that a frog injected with one pituitary gland plus 2 macerated frog adrenal glands ovulated completely in 24 hours. Apparently, then, cortical and androgenic steroids in proper dosage may also facilitate pituitary-induced ovulation *in vivo*.

Summary. In October, when *Rana pipiens* ovaries are notoriously insensitive to ovulatory influences, injection of one frog pituitary gland in combination with small dosages of progesterone induced nearly complete ovulation within 24 hours. Progesterone, and to a lesser extent testosterone, also exerted some ovulatory effect when injected alone.

† Supplied by Dr. P. L. Perlman of Schering Corp.

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Uterine Phosphamidase in Sex Hormone-Treated and Deciduomata-Bearing Ovariectomized Rats.* (26334)

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The enzyme phosphamidase hydrolyzes the N-P bond of amides of phosphoric acid. The naturally occurring phosphamides such as creatine and arginine phosphates, however, are not subject to phosphamidase hydrolysis *in vivo,* but are dephosphorylated by the action of the ATP-transphorylase system. Therefore, no specific metabolic role can be ascribed to this enzyme at present.

Previous reports concerning uterine phosphamidase have been limited to histochemical study of the enzyme in biopsies of human endometrium taken during the menstrual cycle(1,2). The present investigation was undertaken to localize the sites of phosphamidase activity in the uterus of the rat, to determine the amount quantitatively, and to ascertain whether variations in enzyme activity followed simulation of the uterus by steroid sex hormones or occurred during the deciduomal reaction.

Materials and methods. Ten to 24 days following bilateral ovariectomy, 95 virgin female Wistar rats, 90 to 120 days of age, were divided into the following 8 groups of 8 to 20 animals each. Rats in each group were treated as described below: (1) controls—no further treatment, (2) injected with 2 μg of estradiol benzoate† 2 days before sacrifice, (3) injected with 10 μg of estradiol benzoate 2 days before sacrifice, (4) injected daily with 2 mg of progesterone‡ for 3 days before sacrifice. In the remaining 4 groups the uteri were sensitized with progesterone as above and were then traumatized on the fourth day by passing a sterile silk thread longitudinally through the uterine lumen. The hormone injections were continued and the animals were sacrificed at 2, 4, 6 and 8 days after traumatization. Each dose was dissolved in 0.1 ml of a mixture of sesame and olive oils and was injected subcutaneously.

Animals were killed by decapitation after sublethal administration of chloroform. Uteri were removed and divided into 3 portions. The largest was weighed to the nearest 0.1 mg and homogenized in 9 times its weight of distilled water in a cold Potter-Elvehjem apparatus. The second portion was also weighed, then dried to determine the amount of tissue residue. The third portion was fixed in cold ethanol for 24 hours and paraffin sections were subsequently prepared for histochemical localization of the enzyme.

Phosphamidase activity was determined quantitatively by a modification of the method of Holter and Li(3) which utilizes liberation of inorganic phosphorus from N-(p-chlorophenyl) - diamidophosphoric acid. Duplicate 0.3 ml aliquots of the 10% tissue homogenate were added to 0.1 ml of a solution containing 11 mM of substrate,§ 20 mM of sodium hydroxide, 19.2 mM of sodium acetate and 60.8 mM of acetic acid per liter. pH of the substrate solution was 4.6. After 2 hours incubation at 40°C enzyme activity was arrested by adding 2 ml of 10% trichloroacetic acid. Liberated phosphorus was measured by the method of Fiske and Subbarow(4). The results have been expressed as micrograms of phosphorus liberated/10 mg of dry tissue/2 hours incubation at 40°C (Table I).

In addition, sites of enzyme activity were

* Aided by Grant from Nat. Cancer Inst., U.S.P.H.S.

† Progynon—Schering Corp., Bloomfield, N. J.

‡ Progesterone— B and B Medical Supply Co., Cincinnati, Ohio.

§ N-(p-chlorophenyl)-diamidophosphoric acid was purchased from Dajac Laboratories of Borden Co., Philadelphia, Pa. as "p-chloroanilido-phosphonic acid synthesized by the method of Otto." It has been shown that the former rather than the latter compound is obtained by Otto's procedure. Both, however serve satisfactorily as substrates for demonstration of phosphamidase activity.

TABLE I. Uterine Phosphamidase Activity in Sex Hormone-Treated and Deciduomata-Bearing Ovariectomized Rats.

Treatment	No. of assays	Phosphamidase activity*
None	9†	16.9 ± 1.1‡
2 μg estradiol benzoate, 1 dose 2 days before sacrifice	8	15.0 ± .9
10 μg *idem*	8	12.3 ± 1.5
2 mg progesterone, daily for 3 days before sacrifice	8†	9.8 ± 1.6
Deciduomata, 2 days after uterine traumatization	9	20.7 ± 2.8
Idem, 4 days after	10	51.7 ± 4.0
" , 6 " "	9	42.4 ± 4.3
" , 8 " "	8	26.8 ± 3.4

* μg of phosphorus/10 mg of dry tissue/2 hr incubation at 40°C.

† Tissues from 2 or 3 uteri were pooled for each assay.

‡ Mean ± S.E.

determined histochemically in the tissue sections by the method of Gomori(5) as modified by Meyer and Weinmann(6).‖

Observations. Enzyme activity in the animals treated with 2 μg of estrogen did not differ significantly from that in the untreated castrates. On the other hand, the level of activity was significantly depressed in the rats treated with 10 μg of estrogen ($p<0.02$) and with progesterone ($p<0.01$)(Table I). In the uteri containing deciduomata, in all instances phosphamidase activity was greater than in the animals treated with progesterone alone ($p<0.01$ in each case). Progressively higher levels were observed through the fourth day following traumatization—when a peak of more than 5 times the activity in untraumatized progesterone-treated animals was reached. Thereafter there was a progressive decrease in deciduomal enzyme activity.

Histochemical results were not uniform. As previously experienced with the technic (5,6,7), staining varied within individual sections and in consecutive sections taken from the same tissue specimen. The reason for this variability lies in the fact observed by the present authors(8) that less than 8% of the original phosphamidase activity of fresh tissue survives in tissue sections prepared in the prescribed manner. Because of this problem the sites of enzyme activity described below are composite patterns derived through microscopic study of a number of sections from each specimen.

In both the untreated and hormone-treated castrates uterine phosphamidase was confined to the luminal and glandular epithelia of the endometrium and tended to be more concentrated at either the apical or basal pole. There was an inverse relationship between overall intensity of cytoplasmic and nuclear staining. In the deciduomata, on the other hand, in addition to the epithelial activity described above, phosphamidase was present in the differentiating cells of the endometrial stroma. Unlike the epithelial staining, staining in the stromal cells was generally uniform throughout the cytoplasm. However, the same inverse relationship between cytoplasmic and nuclear activity was present. Furthermore, a distinct zonation of activity was apparent in the deciduomata themselves, especially on fourth and sixth days after traumatization. That is, in the deciduomal cells closest to the uterine lumen there was intense cytoplasmic activity, but little or no nuclear reaction. Progressing centrifugally a gradual reversal of staining was observed so that at the periphery of the deciduoma nuclear activity was greatly increased and cytoplasmic activity sharply reduced or absent.

Discussion. Both Remotti(1) and Oehlert, *et al.*(2) observed a pattern of phosphamidase activity in human endometrium which differed from that seen in the rat. They found that during the menstrual cycle enzyme activity was present in the nuclei of the stromal cells as well as throughout the epithelial cells. There was a gradual increase throughout the follicular phase of the cycle which reached a peak during the secretory phase. Late in the latter phase phosphamidase activity declined and disappeared during menstruation. In the rat, on the other

‖ In a personal communication Dr. Julia Meyer suggested further modifications of Gomori's method which have been incorporated. The incubation solution used contained 4.41 mM of substrate (supplied through the courtesy of Dr. Meyer), 3.5 mM of lead nitrate, 45.0 mM of malaic acid and 34.8 mM of sodium hydroxide per liter, and had a pH of 4.1.

hand, both estrogen and progesterone decrease the activity below that present in the uterus of the castrate and the enzyme does not appear in the endometrial stroma unless the latter is first traumatized. It is evident that there is a considerable species difference both in distribution of phosphamidase in the endometrium and in its hormonal regulation.

Meyer and Weinmann(9,10,11) have studied the distribution of phosphamidase in a number of rat organs and have correlated the enzyme activity with the physiological processes known to occur in the same sites. They have found phosphamidase to be associated with (1) active transport and other types of physicochemical work, (2) synthesis of nonprotein substances in both exocrine and endocrine glands, and (3) cellular differentiation. These processes are known to take place in various component tissues of rat endometrium and are largely dependent upon stimulation by estrogen and progesterone. Since the present experiments have demonstrated that both these hormones depress phosphamidase in the castrate rat uterus, the hypothesis of Meyer and Weinmann cannot be supported in this instance. In the deciduomal reaction, on the other hand, where there is a centrifugally progressive differentiation of stromal cells into decidual-like cells, phosphamidase activity approximates that which would be expected on the basis of the experience of Meyer and Weinmann. The present observations reemphasize the paucity of information concerning the metabolic role of phosphamidase and the need for further study of its activity in other tissues and organs.

Summary. The sites of phosphamidase were localized histochemically and amount of enzyme activity measured quantitatively in the uteri of untreated, estrogen- and progesterone-treated, and deciduomata-bearing ovariectomized rats. Enzyme activity was found in the lumenal and glandular epithelia of the endometria of all the animals studied. In addition, phosphamidase was present in the differentiating stromal cells in the uteri in which the deciduomal response had been elicited. Estrogen in high dosage and progesterone depressed the enzyme activity compared with that in untreated castrates. During the development of deciduomata the enzyme level rose to over 5 times that in animals treated with progesterone but not traumatized.

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Antitumor Activity of 1-β-D-Arabinofuranosylcytosine Hydrochloride. (26335)

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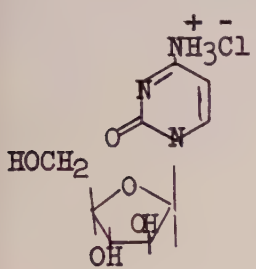
In this laboratory as well as others, investigations of purine and pyrimidine derivatives as potential antimetabolites which might inhibit *in vivo* biosynthesis of DNA and/or RNA have lead to development and clinical study of such compounds as 6-mer-

captopurine(1), psicofuranine(2), 5-fluoro-2′-deoxy-uridine(3), 5′-iodo-2′-deoxyuridine (4), etc.

Preliminary assays in this laboratory revealed that 1-β-D-arabinofuranosylcytosine hydrochloride [I]* exhibited activity against Sarcoma 180. Walwick, Roberts and Dekker(5) reported the preparation of the free base, "3-β-D-arabinofuranosylcytosine." Pizer and Cohen(6,7) investigated the metabolic activity of this base ("Spongocytidine") and related compounds in mutants of 2 strains of *Escherichia coli*. Evidence for the antitumor activity of I against Sarcoma 180, Ehrlich's carcinoma, and L-1210 leukemia is here presented.



(I)

Experimental. 1-(2′,3′,5′-Tri-O-acetyl-β-D-arabinosyl)-uracil(8) was converted into the base of I by a process essentially identical with that of Fox, *et al.*(9), and purified as the hydrochloride (I) which was used in the biological studies described below. The structure of I, in addition to its method of synthesis, elementary analysis, ultraviolet and infrared absorption spectra, was shown by its deamination with nitrous acid to 1-β-D-arabinofuranosyl uracil(8), by paper chromatographic detection of D-arabinose as the sole sugar moiety following the degradation of I according to the procedure of Burke (10), and by conversion of I into a crystalline product the properties of which were in good agreement with those described by Walwick, *et al.*(5), for "3-β-D-arabinofuranosylcytosine."

Female Swiss mice, weighing 18-20 g each, from Upjohn pathogen-free stock were used for Sarcoma 180 and Ehrlich carcinoma studies. L-1210 leukemia was carried in female BDF_1 mice from Diablo Labs., Berkeley, Calif. Sarcoma 180 and Ehrlich carcinoma were transplanted by subcutaneous injection of 500,000 to 650,000 cells into the groin. The drug (I) was given intraperitoneally once daily as indicated. The solid tumors were measured in 2 diameters periodically. Survival and complete regressions of the tumors were recorded. In studies using L-1210 leukemia comparison of mean survival time between treated and control groups was the criterion of activity.

The significance of the number of regressions and no takes was determined using the tables of Mainland and Murray(11). Standard errors were calculated.

Results. The acute LD_{50} of 1-β-D-arabinofuranosylcytosine hydrochloride (I), in either mice or rats, was greater than 1000 mg/kg. The animals were observed for 7 days for evidence of delayed toxicity. The results of tests of I against recently transplanted and established Sarcoma 180 are given in Tables I and II. It was observed that when dosage was begun the day after transplanting the tumor (T_1) and continued for 7 days, a high percentage of the mice showed no tumors 7 days after discontinuing the drug. Continued observation of the mice over a 25-30 day period showed that many of the tumors which did develop regressed completely without becoming necrotic and perforating.

When treatment was delayed until 5 days after transplanting the tumor, although there was no significant difference in number of tumor takes between treated and control groups, a significantly greater number of regressions occurred in the treated group. Preliminary studies showed that the activity of I orally in mice bearing Sarcoma 180 was one-fifth that found by the intraperitoneal route.

Results of studies on mice bearing the solid form of Ehrlich carcinoma (Tables I and II) indicate a similar type of activity. When treatment was delayed for 7 days after transplanting the tumor, the size of the tumors in the treated group was significantly

* Details of the synthesis of I will be reported elsewhere by one of us (J.H.H.).

TABLE I. Effect of 1-β-D-Arabinofuranosylcytosine Hydrochloride on Sarcoma 180 and Ehrlich Carcinoma.

Tumor	Dosage,* mg/kg/day	No. mice	Day of reading†	No. of tumors	Avg T. meas. ± stand. error, mm
S-180	5	10	8	10	9.3 ± .35
			17	10	15.3 ± 1.07
"	10	10	8	5	3.7 ± 1.23
			17	9	10.8 ± 1.41
"	20	10	8	2	1.4 ± .93
			17	4	4.2 ± 1.74
"	50	20	8	0	.0
			17	3	1.4 ± .78
"	—	20	8	20	10.8 ± .30
			17	20	16.4 ± .41
E. carc.	25	20	8	4	1.6 ± .72
			14	10	4.5 ± 1.08
"	—	20	8	20	11.8 ± .39
			14	20	15.9 ± .62

* Treatment for 7 days starting 24 hr after transplanting tumor.
† Days after transplanting tumor.

TABLE II. Effect of 1-β-D-Arabinofuranosylcytosine Hydrochloride on Established Sarcoma 180 and Ehrlich Carcinoma.

Tumor	Dosage, mg/kg/day	Dosage schedule*	No. mice	Day of reading*	No. of survivors	No. of tumors†	Avg T. meas. ± stand. error, mm
S-180	50	$T_5 \rightarrow T_{12}$	20	T_8	20	11	4.48 ± .95
				T_{13}	20	12	4.98 ± .94
				T_{35}	19	4 (11)	3.47 ± 1.68
"	—	—	20	T_8	20	16	7.28 ± .90
				T_{13}	20	20	12.70 ± .60
				T_{35}	19	16 (3)	19.08 ± 2.28
"	50	$T_7 \rightarrow T_{13}$	20	T_8	20	20	10.72 ± .42
				T_{10}	20	19	9.97 ± .68
				T_{33}	18	8 (10)	7.50 ± 2.25
"	—	—	20	T_8	20	20	10.77 ± .30
				T_{10}	20	20	13.25 ± .38
				T_{33}	20	20 (0)	22.23 ± 1.03
E. carc.	50	$T_5 \rightarrow T_{12}$	20	T_8	20	15	7.50 ± 1.04
				T_{14}	20	13	6.40 ± 1.12
				T_{33}	19	10 (6)	8.47 ± 2.23
"	50	$T_7 \rightarrow T_{14}$	20	T_8	20	17	8.98 ± .93
				T_{14}	20	19	11.58 ± .80
				T_{33}	19	16 (3)	12.50 ± 1.71
"	—	—	20	T_8	20	20	11.75 ± .39
				T_{14}	20	20	15.92 ± .62
				T_{33}	19	18 (1)	23.84 ± 2.21

* Subscripts indicate days after tumor implantation.
† No. of complete tumor regressions is given in parentheses.

smaller than the controls ($P = 0.01$).

1 - β - D - Arabinofuranosylcytosine hydrochloride when given to BDF_1 hybrid mice bearing the ascitic form of L-1210 leukemia produced a 260% increase in mean survival time when treatment was started on T_1. When start of treatment was delayed to the fourth day (T_4), a 12.5% increase in survival time was noted (Table III). The mice treated with the nucleoside starting on the fourth day showed complete absence of the ascitic fluid usually found in the controls and no gross evidence of solid tumors in and around the mesentery. Preliminary studies of the leukocyte and differential counts of treated L-1210 leukemic mice indicate that 1-β-D-Arabinofuranosylcytosine hydrochloride caused a leukopenia which was not pres-

TABLE III. Effect of 1-β-D-Arabinofuranosylcytosine Hydrochloride on L-1210 Leukemia.

Dose	Dosage schedule	No. mice	Avg body wt, g	50% surv., days	Time + range, days
50 mg/kg	$T_1 \rightarrow T_7$	10	19.1	18.0	18– *
Saline controls		10	18.2	7.0	7– †
50 mg/kg	$T_4 \rightarrow T_{11}$	10	19.7	9.0	8–15
Saline controls		10	19.9	8.0	7–12

* 2 mice survived for 28 days. All deaths on either 18th or 19th days.
† *Idem* . All deaths on either 7th or 8th days.

ent in treated nonleukemic mice. In L-1210 leukemic mice, average leukocyte counts were 15,500 cells per cu mm (range 9,750-23,750) when treatment was initiated on T_4. On T_{11}, the average leukocyte counts of 4 treated mice were 1,810 cells per cu mm (range 750-3,750) and that of 5 controls 52,000 cells per cu mm (range 18,250-88,000). In contrast, in 5 normal treated mice the leukocyte counts averaged 17,400 cells per cu mm (range 9,000-24,500) on the initial day and 12,600 cells per cu mm (range 6,500-17,300) after 7 days of treatment. In both leukemic and normal mice, number of polymorphonuclear neutrophils decreased more than other cells.

Twenty-five or 50 mg/kg of I given to rats bearing Walker 256, Murphy-Sturm and Guerin tumor for 10 days starting 24 hours after transplanting the tumor produced no tumor inhibition and did not affect normal growth of the rats.

The nucleoside (I) was relatively inactive as a cytotoxic agent against KB cells in tissue culture or when Sarcoma 180 or Ehrlich carcinoma cells were held at room temperature with the compound in the medium for 30 minutes prior to implantation.

Discussion. 1-β-D-Arabinofuranosylcytosine hydrochloride has been shown to be active against Sarcoma 180, E. carcinoma and L-1210 leukemia in experimental mice. The supply of material has limited testing of the compound at higher dosage levels in the tumor-bearing rat. The results in the 2 species are similar to those observed by Welch(12) using 5-iodo-2′-deoxyuridine.

It was considered possible that the antitumor activity of I might have resulted from deamination *in vivo* to 1-β-D-arabinofuranosyluracil. However, a preliminary test using the latter compound at 100 mg/kg/day for 7 days against a recently transplanted S-180 showed only weak activity. Studies on the mechanism of action of I are in progress.

Summary. 1-β-D-Arabinofuranosylcytosine hydrochloride has been shown to be active against recently transplanted and established Sarcoma 180, Ehrlich carcinoma and L-1210 leukemia in mice. This nucleoside was inactive at the same dose per kilogram of body weight in tumor-bearing rats.

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Permeability of Rat Adrenals *in vitro* to D-Xylose in Presence and Absence of ACTH.* (26336)

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Previous work *in vivo* has shown that the "non-utilizable" pentose, D-xylose, is excluded from a major fraction of the cell water of the adrenal of hypophysectomized rats, and that administration of adrenocorticotrophic hormone (ACTH) specifically increased distribution of D-xylose in adrenal to a level approaching that found in stressed intact rats(1). These findings suggest that the penetration of sugar into the adrenocortical cell may be the rate-limiting step for glucose metabolism *in vivo,* and raise the possibility that the physiological effects of ACTH may be secondary to its influence on intracellular availability of substrate. In attempting to evaluate the latter possibility, we have examined the effects of ACTH upon D-xylose permeability in sectioned rat adrenals *in vitro,* which exhibit the well-established(2) increase in corticosteroidogenesis following addition of exogenous ACTH. It has been found that sectioned adrenals studied *in vitro* exhibit altered permeability characteristics in that D-xylose rapidly enters a major fraction of adrenal cell water in absence of ACTH; under these *in vitro* conditions, ACTH has no significant effect on sugar permeability but stimulates corticosteroidogenesis.

Methods. Intact or hypophysectomized (2 days) rats of the Sprague-Dawley strain, weighing 150 to 200 g, were employed. Following decapitation, adrenals were removed, bisected, and apportioned for incubation so that each flask contained half of each adrenal from 2 rats, giving a series of paired flasks containing tissue equivalent to 2 adrenals. In most cases, adrenals were preincubated for 1 hr in a gyratory shaker at 38°, under O_2 (5% CO_2), in 3 ml of Kreb-Ringer bicarbonate medium, containing glucose (1 mg/ml). For the final incubation, the preincubation medium was decanted and replaced with fresh medium containing labeled D-xylose and inulin. In all experiments, tracer concentrations were .025 μc/ml of D-xylose-1-C^{14} (National Bureau of Standards, S.A. 2.26 μc/mg) and .03 μc/ml of inulin-carboxyl-C^{14} (New England Nuclear, S.A. 3.0 μc/mg). One of each pair of flasks received 750 mU Corticotrophin A (Armour) in 0.1 ml N/100 HCl-0.9% saline; controls received 0.1 ml of vehicle. After various intervals of incubation ranging from 5 to 120 min, adrenals were removed, blotted in a uniform manner and weighed.

D-xylose and inulin were extracted from the adrenal tissue in a minimum volume of water (.4 ml) by heating in a boiling water bath(1), and the radiotracers from tissue and media were separated by chromatography on #2 Whatman paper for 2 days, using 95% ethanol as a mobile phase. In this system, inulin remains at the starting line and xylose is quantitatively recovered in the overflow flasks. D-xylose and inulin content of incubated adrenals and media were thus determined separately by plating these fractions and counting in a gas flow chamber.

The results with D-xylose and inulin are presented in terms of volume of distribution in total tissue, expressed as percent of total tissue wet weight in equilibrium with the medium (100 x cpm/g tissue per cpm/ml medium). It has been assumed that inulin is a measure of the extracellular compartment, and does not penetrate into the cell water; by measure of total water content of incubated tissues and inulin space, it is therefore possible to calculate the apparent distribution of D-xylose in cell water.

Corticoids in the medium after incubation were determined by the fluorimetric method of Moncloa *et al.*(3).

Results. Table I shows results in terms of (a) volume of distribution of either D-xylose or inulin in total tissue, (b) calculated volume of distribution of D-xylose in intracellu-

* This work has been supported by grants from U. S. Public Health Service, Nat. Science Foundation, and Commonwealth Fund.

TABLE I. Influence of ACTH on Distribution of Tracers and Corticoid Production in Adrenal Tissue of Pituitary Intact and Hypophysectomized Rats *In Vitro*.

Type rat used	Preincubation	Period of incubation (min.)	Vol of distribution of tracers					D-xylose distribution in I.C.W.*		Corticoid (μg/100 mg wet tissue)	
			D-xylose		Inulin						
			Control	ACTH	Control	ACTH		Control	ACTH	Control	ACTH
Pituitary intact	1 hr	5	36 ± 3.6 (6)	41 ± 3.0 (6)	14 ± .7 (6)	17 ± 1.0 (6)		35	40	1.4 ± .2 (6)	3.0 ± .6 (6)
		15	47 ± 2.5 (13)	48 ± 2.3 (9)	22 ± 1.2 (13)	22 ± 1.2 (9)		46	47	1.5 ± .2 (9)	4.0 ± .5 (9)
		30	48 ± 6.7 (4)	55 ± 5.2 (4)	20 ± 3.8 (4)	31 ± 2.3 (4)		49	52	2.8 (2)	8.3 (2)
		60	61 ± 2.0 (18)	61 ± 2.3 (11)	29 ± 3.7 (4)	33 ± 2.3 (4)		67	65	6.3 ±1.0 (9)	18.6 ±4.2 (9)
		120	62 ±11.4 (4)	60 ± 4.1 (4)	28 ± 2.3 (4)	37 ± 3.8 (4)		69	59	5.3 ± .2 (4)	22.9 ±7.7 (4)
Hypophysectomized	1 "	15	41 ± 3.4 (4)	48 ± 2.7 (4)	19 ± 1.6 (4)	25 ± 1.8 (4)		38	44	1.3 ± .3 (4)	2.4 ± .4 (4)
		60	57 ± 3.6 (4)	69 ± 8.1 (4)	22 ± 1.1 (4)	32 ± 3.1 (4)		63	82	2.1 ± .3 (4)	13.1 ±1.4 (4)
	None	15	62 ± 3.6 (4)	66 ± 2.4 (4)	17 ± 2.9 (4)	16 ± 1.9 (4)		76	83	1.0 ± .1 (4)	1.2 ± .3 (4)
		60	75 ± 1.2 (4)	72 ± 5.1 (4)	19 ± 2.2 (4)	19 ± 1.7 (4)		96	92	1.4 ± .2 (4)	12.1 ± .2 (4)

Values are mean, stand. error, and No. of observations.

* Intracellular water is taken to be the difference between total tissue water (76.6% ± 0.7, 10 observations) and inulin space, assuming that inulin distributes exclusively in the extracellular compartment; ACTH had no detectable effect on water content of incubated adrenals.

lar water, and (c) corticoid production. In the group using adrenals from pituitary-intact rats, D-xylose distribution increases with the interval of incubation up to 60 min, but does not increase further by 120 min. After 5 min incubation, volume of distribution of D-xylose is about two-thirds that at 60 min, indicating a very rapid entry of D-xylose. No significant effect either on rate or final plateau level of D-xylose distribution as a result of ACTH addition was detected. The results further indicate that the inulin space increases with time of incubation, and that ACTH tends to increase the inulin space particularly at 30 to 60 min. A corticosteroidogenic response to ACTH is observed, being clearly evident in 30 min and maximal in 2 hr.

The results on D-xylose and inulin using adrenals from hypophysectomized animals are similar to those in adrenals from pituitary-intact rats, provided that the adrenals of hypophysectomized rats are also preincubated. Without initial preincubation, the D-xylose space is somewhat larger and the inulin space smaller than in preincubated adrenals from either pituitary-intact or hypophysectomized rats. No appreciable effect of preincubation was observed on corticoid production nor on corticoid response to ACTH in adrenals from hypophysectomized rats.

Discussion. These studies establish that sectioned rat adrenals, which respond to added ACTH with increased corticoid production, exhibit marked alterations in permeability relationships relative to those existing *in vivo*. In absence of ACTH, D-xylose is excluded from 70 to 75% of the cell water of the adrenal *in vivo* and ACTH increases the distribution of D-xylose in cell water to almost 70% (1). *In vitro,* however, in absence of added ACTH, D-xylose rapidly enters and distributes in about 70% of the intracellular water of the sectioned adrenal of hypophysectomized or normal rats. Under these circumstances, the finding that *in vitro* addition of ACTH had no significant influence on D-xylose penetration, is not unexpected since the permeability bar-

riers operative *in vivo* do not appear to be present under these *in vitro* conditions. Similarly, inulin space measured *in vitro* is altered from the *in vivo* situation. *In vivo,* inulin space is about 15% of total wet weight of the tissue, while *in vitro* inulin space after preincubation plus one hour incubation is 29% in adrenals from pituitary-intact rats and 22% in those from hypophysectomized rats. Addition of ACTH had no effect on inulin space in glands not preincubated, but tended to increase the inulin space in preincubated glands. With the number of animals employed, the differences in inulin space between control and ACTH are not statistically significant in the series of adrenals from normal animals, but in the group from hypophysectomized rats, preincubated 1 hr, the difference is significant ($P<0.05$). Although we have assumed for purposes of calculating intracellular distribution of D-xylose that the increased inulin space observed as incubation time increases represents an expansion of the extracellular volume, this increase might represent the entry of inulin into some of the adrenal cells. Likewise, the tendency for ACTH to increase inulin space under certain conditions may be interpreted in terms of either alternative.

The alteration of sugar permeability exhibited by bisected rat glands *in vitro* suggests that a large number of cells in the preparations are damaged, whether as a result of relative anoxia suffered by cells in the interior of the tissue section, a deficiency in the medium employed, or to other, as yet unknown, factors involved in the technic. Whatever the basis, it also involves other aspects of cell maintenance, since we have found that intracellular K^+ is lost, and Na^+ is gained by sectioned adrenals *in vitro;* thus, *in vivo,* Na is excluded and intracellular K^+ is about 170 μM/ml cell water, whereas following incubation, the K^+ is reduced to 98 μM/ml cell water, and there is an approximately corresponding gain of Na^+ (unpublished observations). Moreover, *in vitro,* ascorbic acid(4), and even intracellular enzymes are liberated into the medium(5). It is thus apparent that sectioned rat adrenal studied *in vitro* exhibits properties which are significantly different from those which are manifest *in vivo.* The fact that such preparations respond to ACTH with respect to steroidogenesis indicates that the *in vitro* effect of ACTH in this preparation does not appear to be secondary to an increase in substrate availability. Peron and Koritz(6) and Schonbaum *et al.*(5) advanced a similar conclusion from other data. Under physiological conditions, however, where the availability of substrates *is limited* by permeability barriers, the effect of ACTH to modify sugar permeability is probably an important aspect of ACTH action.

Summary. Studies on the permeability properties of bisected rat adrenals, incubated *in vitro,* have revealed marked differences from those which obtain *in vivo.* Thus, in absence of ACTH *in vivo* D-xylose is excluded from a major fraction of the cell water, but *in vitro* D-xylose rapidly penetrates and appears to equilibrate in about 70% of the cell water. *In vivo,* ACTH increases sugar distribution, but under *in vitro* circumstances, where a corticosteroidogenic response is observed, ACTH has no significant influence on rate of penetration or intracellular distribution of D-xylose. ACTH does, however, tend to increase inulin space of sectioned rat adrenal *in vitro.* While these findings suggest that the steroidogenic effect of ACTH *in vitro* is not dependent upon hormonal regulation of sugar availability, the possibility remains that an important aspect of ACTH action *in vivo,* where permeability barriers are operative, involves regulation of sugar permeability.

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An Attempt at Production of Autoimmunity to Tissue of the Gastrointestinal Tract. (26337)

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The existence of autoimmunity as a factor in human disease has been demonstrated in cases of Hashimoto's struma(14,11,1). The production of disease in animals by technics of immunization utilizing tissue antigen to produce disease of the corresponding tissue has been successful in the case not only of thyroid but also of the uveal tract(3), brain (8,6), testes(5) and adrenals(4). The lesions induced have been inflammatory reactions of the tuberculin type(13). This type of reaction, and the rather special tissues in which it has occurred, suggests a relationship with the phenomenon of homograft rejection. It is as though the tissue involved becomes for the first time immunologically "recognized" through the mechanism of the immunization procedure and is then rejected(13). It has been tempting to apply such mechanisms to other human diseases characterized, as in Hashimoto's struma, by chronic inflammation in the absence of demonstrable pathogenic organisms. Among such conditions regional enteritis and ulcerative colitis in particular have been considered by many workers(8,12), as being produced by immune processes. Colitis has been induced in rabbits by producing repeated responses of the Auer or Arthus type(7), and in patients with chronic ulcerative colitis circulating antibody to a polysaccharide antigen obtained from colon has been demonstrated(2,9). We are not aware, however, that a serious attempt has been made to produce autoimmunity to material from the gastrointestinal tract using the technics which have been successful in the case of thyroid, eye, brain and testes. In this communication, we are reporting an attempt in which tissue from 3 levels of the gastrointestinal tract, made up as a whole tissue antigen in Freund's adjuvant, was used in immunization by intradermal injection of several animal species.

Materials and methods. Animals. New Zealand white female rabbits (5-6 lb) Western white female rats (200 g), laboratory-bred white A-strain mice (20 g) and white Hartley strain guinea pigs (300 g) were used in this study. All animals were kept in wire cages and fed chow and water *ad libitum*.

Antigens. Normal animals of each species were sacrificed to provide antigen. The entire digestive tract was dissected free and portions of stomach, small bowel, and large bowel were removed, opened and washed free of contents. They were immediately chilled in ice water and ground in a high speed Virtis grinder at 45,000 rpm with a sufficient amount of saline to give a fluid suspension. Portions of these saline suspensions were emulsified in 2 volumes of light mineral oil-Arlacel* mixture (8.5 to 1.5) containing 10 mg/ml of killed mycobacterium tuberculosis. Final concentrations of tissue in emulsion for each preparation are given in Table I.

Injections. All animals were given approximately 0.02 ml of the appropriate emulsion into each food-pad (or several toe pads on each foot in the case of the rabbits). In addition, rabbits, guinea pigs and rats were given 2-3 intradermal injections of 0.02 ml of adjuvant in the flanks.

An additional 3 rabbits were injected every 2 weeks with the rabbit colon emulsion for a period of 10 weeks, when they were sacrificed.

All animals were weighed periodically and carefully watched for signs of fecal blood and general distress. At 2 weeks, 4 weeks and 8 weeks, pairs of animals from each group were sacrificed and the gastrointestinal tract was dissected free and examined for gross pathology. Then several portions of stomach, small and large intestine were removed, fixed in formalin and sectioned for

* Obtained from Atlas Powder Co., Wilmington, Del.

TABLE I. Animals and Antigens Used for Immunization Studies.

Animals	No.	Antigen used	Conc. of tissue in adjuvant mixture, mg/ml	ml inj. /animal
Rabbit	6	Rabbit stomach	50	.2
"	"	" small bowel	40	.2
"	"	" large "	50	.2
G. pig	"	G. pig stomach	40	.14
"	"	" small bowel	50	.14
"	"	" large "	50	.14
Rat	"	Rat stomach	30	.14
"	"	" small bowel	40	.14
"	"	" large "	50	.14
Mouse	"	Mouse stomach	3	.08
"	"	" small bowel	50	.08
"	"	" large "	60	.08
Rabbit	3	Rabbit large bowel	220	.2 ml repeated 4 times

histologic examination with haematoxylin and eosin stain.

Results. Table I summarizes the data on antigen preparations used and amounts injected in the various species. None of the animals developed gross signs of pathology. All gained weight normally during the experiment and showed no evidence of blood in the stools either visually or by benzidine test. Grossly, the gastrointestinal tracts looked normal with no evidence of ulceration, hemorrhage or inflammation. Histologically, no signs of pathology such as necrosis, infiltration or hemorrhage were apparent. In the small bowel of all guinea pigs were large masses of monocytes but these appeared to be consistent with normally distributed lymphoid tissue (Peyer's patches) and were not infiltrating.

In the present experiment, therefore, no evidence of pathology was found in rabbits, guinea pigs, rats and mice injected with homologous stomach, small intestine and large intestine incorporated into Freund's adjuvant.

Discussion. This negative result cannot of course be construed as final evidence that autoimmunization to tissue from the gastrointestinal tract cannot be produced experimentally. Quantity of antigen, frequency of injection and length of observation period could all be varied with conceivably different results. The many reports in the literature, however, that autoimmunity to brain, thyroid and testes, as well as other tissues, may be produced readily in at least one of the species studied by this method and route of immunization, makes it appear more likely that fundamental immunologic restrictions rather than shortcomings in technic account for the present negative results.

It has been suggested(10) for example, that the tissues towards which autoimmunity has been succesfully produced seem to have in common some degree of separation by more or less impermeable barriers from the general vascular bed. Thus dissemination of the antigen to sites of immune response would be inhibited by the pia-glia membrane of the central nervous system, the capsule of the lens of the eye, the basement membrane of the seminiferous tubules and the follicle epithelium of the thyroid. In this event, tolerance to these tissues would not be achieved in the young animal, and subsequent exposure to them would produce autoimmunization. It is doubtful whether this would hold true for the elements of the gastrointestinal tract.

Summary and conclusions. Immunization of rabbits, guinea pigs, rats, and mice with antigen in Freund's adjuvant prepared from homologous stomach, small intestine and colon was not found to be pathogenic to the animals as to production of gastrointestinal disease.

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Histochemistry LXIV. Succinic Dehydrogenase System in Isolated Mast Cells from the Rat.* (26338)

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Growing recognition of the important physiological role of mast cells prompted this study on a succinic dehydrogenase system which could reflect certain metabolic conditions in the cells as a function of the influence of various states and agents. Previously, in a study of mast cells isolated from peritoneal washings from the rat, *in vitro* effects on the cell morphology of agents, known or suspected of having physiological influences on these cells, were reported(1). This investigation is concerned with effects of age, environmental stress (cold), and subcutaneous injections of ACTH, *E. coli* endotoxin, histamine, serotonin, and compound 48/80 ("histamine liberator") on the activity of the enzyme system in the isolated cells.

Methods. Male albino rats, Sprague-Dawley (Holtzman, Madison, Wis.), were housed singly or in pairs in a constant climate room (25° ± 1°C, 30-50% relative humidity, controlled illumination, lights on 6 a.m. and off 6 p.m.) for at least 4 days prior to use. All rats, except those employed for study of age effects, were approximately 3 months old and weighed about 300 g. They received Purina Fox Chow and tap water *ad libitum*, and were killed instantly between 11:00 and 11:30 a.m. by a single blow on the head.

Suspensions of pure mast cells in Hanks' solution were obtained from peritoneal washings as described by Glick *et al.*(2) using a sucrose density gradient. After this work was started, Ficoll (Pharmacia, Uppsala, Sweden), a dextran that provides solutions of the required density with relatively low viscosity, became available, and its value for density gradient separations was shown(3). Mast cells isolated in a Ficoll gradient are not subjected to as great an osmotic strain and they retain their histamine which is lost to some degree during separation in sucrose (4). Comparison of the activity of the succinic dehydrogenase system in cells isolated in either medium showed no difference however (Table I). Apparently the enzyme in the mitochondria is unaffected or influenced to the same degree by either procedure, although greater morphological damage was observed in cells separated in the sucrose gradient.

After isolation, washing the cells in Hanks' solution had no morphological effect, but in a Versene-phosphate buffer (0.067 M phosphate, 5×10^{-4} M Versene), of the pH (7.7) required for optimal enzyme activity, the cells were all disrupted, resulting in marked increase in activity (Table I).

The activity of the succinic dehydrogenase

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† Data in this paper were presented in a thesis submitted by S. P. in partial fulfillment of the requirements of the Graduate School for the degree of Master of Science in Physiological Chemistry.

TABLE I. Influence of Isolation Technic on a Succinic Dehydrogenase System in Peritoneal Mast Cells from the Rat.

Technic	Enzyme activity (mμM INPT formazan/μg protein-nitrogen/hr) Mean*	S.D.	S.E.
Sucrose density gradient†	1.7	.20	.12
Ficoll " " ‡	1.7	.35	.20
Versene-phosphate wash†§	1.9	.28	.07
Hanks' solution wash§ (cells intact)	.3	.07	.04

* 3 experiments on separate rats.
† Cells disrupted by Versene-phosphate wash.
‡ 33.4% Ficoll dialyzed and concentrated to half vol (sp. gr. 1.11), density gradient made with 3 dilutions: 1.3, 2.0, 3.3 times, giving 1.07, 1.05, 1.02 sp. gr., respectively.
§ Cells isolated in Ficoll density gradient.

system was determined by modification of the method based on reduction of INPT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5 phenyl tetrazolium chloride](5). Optimal conditions for measurement of activity in mast cells were elaborated, and concentration of sample and digestion time were chosen to fall within range of a linear relationship with activity. The final procedure followed was: 1. Place 60 μl of buffer-substrate solution. (0.3% INPT in 0.033 M phosphate buffer, pH 7.7, and 0.33 M sodium succinate) in each of 3 reaction tubes (glass-stoppered, 200 μl capacity), and 60 μl of blank solution (buffer-substrate solution without the succinate) in each of another 3 tubes. 2. Add 20 μl of mast cell suspension to each of the tubes, stopper and place them in a 37°C bath for 1 hour. 3. Transfer tubes to boiling water for 1 min, centrifuge at 100 × g for 2 min, suck off supernatant fluid and dry residues (protected from light) overnight *in vacuo* over calcium chloride. 4. Dissolve formazan product in 50 μl of ethanol-tetrachloroethylene (3:1 by vol.) by mixing, letting stand for 1 hour in the dark and mixing again. 5. Cover tubes with Parafilm, centrifuge at 1000 × g for 1 min, transfer total supernatant fluids to 50 μl cuvettes, and measure absorbance at 505 mμ (Beckman DU spectrophotometer). 6. Calculate results from standard formazan curve.

The precision of the method was established by analysis of 10 samples of a given suspension of mast cells which gave a mean value of 1.67 mμM formazan/μg protein-nitrogen/hour, 0.065 std. dev., 0.021 std. error, 3.9% coefficient of variation.

Protein-nitrogen was determined by the bromsulfalein method(6).

Results. Although changes with age in number and morphology of peritoneal mast cells from the rat have been reported(7), no demonstrable effect of age on enzyme activity was found over the range 30-120 days (Table II).

In rats kept at 5°C for 1 week, increased numbers of tissue mast cells in a 1.5-2-month-old group and decreased numbers in an older (>1 year) group were observed(8). Prolonged exposure (2-4 weeks) at 2° or 6°C caused increased numbers in perivascular regions and decreased numbers, which gradually returned to initial values, in intervascular mesenteric areas of 300 g rats(9). In the 3-month-old, 300 g, rats used in this work, cold stress over short periods increased activity to a degree related to time of exposure, and injection of *E. coli* endotoxin also caused elevation. Therefore, it is not surprising that ACTH (15 mg/kg) increased activity with further increase at higher dose (25 mg/kg). But under the experimental conditions no change was observed with 10 mg/kg (Table III). With doses above 25 mg/kg, only disrupted mast cells could be isolated from the peritoneal washings. In all cases of elevated activity from treatments, a considerable proportion of disrupted cells were seen in the fractions isolated. These findings are in accord with evidence that stress conditions, ACTH stimulation of the

TABLE II. Effect of Age of Rat on a Succinic Dehydrogenase System in Peritoneal Mast Cells.*

Age (days)	No. analyses on separate rats	Enzyme activity (mμM INPT formazan/μg protein-nitrogen/hr) Mean	Std. dev.	Std. error
30	4	1.5	.17	.09
60	6	1.6	.19	.10
90	7	1.4	.21	.08
120	4	1.7	.16	.08

* Isolated in sucrose density gradient.

TABLE III. Influence of Treatments on a Succinic Dehydrogenase System in Peritoneal Mast Cells from the Rat.

Treatment*	Hours between inj. and killing	No. rats	Enzyme activity (mμM INPT formazan/μg protein-nitrogen/hr) Mean	Std. dev.	Std. error	p†
Control, 25°C		3	1.6	.07	.04	
4°C, 20 min.		4	2.1	.70	.35	>.2
4°C, 60 "		5	2.5	1.10	.49	>.1
4°C, 180 "		6	4.1	.95	.39	.01
Control	3	7	1.9	.33	.13	
10 mg ACTH/kg	3	9	1.9	.24	.08	.5
Control	3	4	1.2	.20	.10	
15 mg ACTH/kg	3	11	1.5	.49	.66	.01
Control	3	3	1.7	.19	.11	
25 mg ACTH/kg	3	6	2.7	.58	.24	.01
Control	2	4	1.2	.06	.02	
5 mg *E. coli* endotoxin‡/rat	2	8	2.6	.40	.14	.01
Control	1	4	1.4	.25	.13	
.5 mg 48/80§/kg	1	8	2.5	.62	.13	.01
Control	1	5	1.6	.26	.12	
1 mg histamine/rat	1	9	1.5	.30	.10	>.5
6 " " "	1	3	1.8	.48	.16	>.5
Control	1	5	1.7	.27	.12	
1 mg serotonin/rat	1	9	1.6	.40	.13	>.4
5 " " "	1	3	1.9	.20	.12	>.4

* Subcut. injections in 1 ml physiological saline solution, control injections of 1 ml of the saline solution. Cells isolated in sucrose density gradient.

† Significance of difference from control.

‡ Prepared and supplied by Dr. Wesley Spink, Dept. Medicine, Univ. Minnesota.

§ (Burroughs Wellcome) supplied by Dr. Edwin J. deBeer, Tuckahoe, N. Y.

adrenal, or adrenal cortical hormones cause disruption of mast cells under certain conditions (*e.g.*, 10, 11, 12). However, since all cells are disrupted by the Versene-phosphate buffer prior to measurement, the increased activities observed can not be ascribed to this factor. The increase in activity per unit protein-nitrogen may be due to an absolute increase in amount or state of activity of the enzyme system, to enhanced accessibility of enzyme to substrate because of a factor such as greater permeability of mitochondrial membranes, or to both. Evidence has been presented that mitochondria in mast cells are distinct from the granules containing histamine, heparin and serotonin(13).

Conceivably, loading the body with histamine or serotonin, both of which are synthesized in rat mast cells, might reduce their production with lowering of certain metabolic activities of the cells and decreased succinic dehydrogenase activity. The present work, admittedly not critical in settling this question, at least lends no support to the possibility, since injection of either histamine or serotonin had no significant effect on the enzyme activity as measured (Table III).

Summary. Isolation of mast cells from peritoneal washings of the rat in either a sucrose or Ficoll density gradient gave the same activity of a succinic dehydrogenase system in the cells. Disruption of isolated cells by washing them with a Versene-phosphate buffer (pH 7.7) greatly elevated the enzyme activity. Cold stress or subcutaneous injection of ACTH, *E. coli* endotoxin, or compound 48/80, increased activity per unit protein-nitrogen significantly, and independently of gross cell disruption, since all cells were disrupted for measurement. No effects on activity of age from 30 to 120 days, or of subcutaneous injections of histamine or serotonin were observed.

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Effects of Pituitary Removal or Transplantation on Ovarian Ascorbic Acid Depletion in the Rat.* (26339)

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It is now accepted that luteinizing hormone of the pituitary (LH), when given intravenously to suitably prepared immature rats, will deplete ovarian ascorbic acid. This procedure forms the basis for a sensitive assay method for LH(1) and it has been reported that the other anterior lobe hormones are without effect on this response(1,2). Briefly, the assay animals are prepared by treating 25-26-day-old female rats with exogenous gonadotrophins to induce precocious ovulation and obtain animals with heavily luteinized ovaries. It has been shown previously that, following ovulation, such animals remain in a state of pseudopregnancy for 10-12 days as judged by vaginal smears, decidual reactions and the Hooker-Forbes test for plasma progesterone(3). Furthermore, it must be presumed that luteal function in these immature animals is promoted by pituitary luteotrophin secretion. It is not known, however, to what extent the ovarian response to LH (ascorbic acid depletion) may be conditioned or modified by other circulating pituitary hormones. It has been reported that a rapid loss of ovarian ascorbic acid follows hypophysectomy and that this is attended by a decreased sensitivity to LH (4). Since it has been shown that pituitary autografts in the kidney are capable of maintaining luteal function in the adult rat(5) it seemed of interest to study the response of ovarian ascorbic acid to LH in immature, pseudopregnant rats after hypophysectomy and hypophysectomy followed by grafting the pituitary under the renal capsule. In the latter situation it was anticipated that a continuous luteotrophin secretion by the graft would maintain the corpora lutea functional for extended periods of time. At the same time the ovaries of the autograft-bearing rats would be removed from any endogenous source of follicle stimulating hormone (FSH) or luteinizing hormone (LH) since it has been found that pituitary grafts in the kidney are incapable of secreting these hormones(5,6).

Methods. Holtzman female rats, 25 days of age, were injected (sbc) with 50 IU of pregnant mares' serum gonadotropin (Equinex).§ This was followed by an injection

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TABLE I. Changes in Ovarian Weight and Ascorbic Acid Concentration in Immature, Pseudopregnant Rat* Following Hypophysectomy or Pituitary Autografting.

Animal group	Treatment		No. of rats	Ovarian wt (mg) †	Ascorbic acid (mg/100 g)
1	Intact, pseudopregnant 6 days		24	115.8 ± 4.5‡	85.7 ± 5.0
2	Pseudopregnant 6 days + hypex.	1 wk	16	85.6 ± 5.1	85.1 ± 4.9
3	*Idem*	2 "	7	95.8 ± 5.1	92.8 ± 6.3
4	"	4 "	14	84.0 ± 4.6	71.6 ± 4.3
5	"	5 "	13	69.7 ± 3.3	85.4 ± 2.9
6	Pseudopregnant 6 days + autografts	1 "	15	117.9 ± 6.6	89.4 ± 5.2
7	*Idem*	2 "	8	123.1 ± 6.7	78.6 ± 7.0
8	"	4 "	15	102.3 ±10.3	58.2 ± 3.2

* All animals were made pseudopregnant by inj. of PMS and HCG.
† Single ovary wt.
‡ Stand. error.

of 25 IU of human chorionic gonadotropin‖ (HCG) 64 hours later. Six days after HCG injection some of the rats were studied as intact, pseudopregnant animals and others were hypophysectomized. The hypophysectomy operation was done by the usual parapharyngeal approach using ether anesthesia and without tracheal cannulation. In several groups of animals the pituitary was removed and quickly autotransplanted to the renal capsule. Following surgery the animals were given a 5% solution of dextrose to drink and the routine diet of Purina laboratory chow was supplemented at intervals with fresh carrots and lettuce. The first objective was to determine the changes with time after operation in ovarian weight and ascorbic acid level. These data are shown in Table I. At autopsy the ovaries were removed, dissected free of uterine tube and connective tissue and weighed on a torsion balance. Ascorbic acid analyses were carried out by a scaled up version of the micromethod described by Lowry *et al.*(7). In other groups of animals prepared in the same manner the influence of hypophysectomy or pituitary autografting on responsiveness of ovarian ascorbic acid to LH depletion was studied (Table II). Four hours prior to autopsy the animals were anesthetized with ether and the left ovary was removed surgically. A standard dose of LH¶ (3.2 μg/100 g) was then injected into a tail vein. Preliminary studies in our laboratory had shown that this dose of LH would induce a maximal depletion of ovarian ascorbic acid when given to pseudopregnant rats in accordance with the conditions of the Parlow assay. At autopsy of these animals the right ovary was removed and prepared for ascorbic acid analysis. In all hypophysectomized animals the sellar region was examined carefully for pituitary remnants. Body weight records were also available and observations of hair texture were made(8). Animals were discarded if evidence suggesting incomplete removal of the pituitary gland was found. In 3 groups of animals studied (Table II, groups 2, 6, 9) one ovary had been removed prior to their use in this study so that no "before LH" ascorbic acid value is shown. Except for these 3 groups the change in ovarian ascorbic acid produced by LH injection is represented in Table II as "% change." In all cases where it was considered desirable to compare mean differences the standard "t" test for significance was applied.

Results. The results are shown in Tables I and II. For the 4 week period studied the ovarian weight is well maintained by the pituitary autografts (Table I). There is, by

‖ Kindly supplied by Dr. L. T. Blouin, Parke Davis and Co., Ann Arbor, Mich.

¶ This preparation was kindly supplied by Prof. C. H. Li, Hormone Research Lab., Berkeley, Calif. It carried the designation Sheep ICSH but because of the nature of this study we prefer to use the alternative designation of Luteinizing Hormone (LH).

TABLE II. Effect of Hypophysectomy or Pituitary Autografting on Ovarian Ascorbic Acid Depletion by LH in Immature, Pseudopregnant Rat.*

Animal group	Treatment		No. of rats	Ovarian ascorbic acid (mg/100 g) Before LH†	After LH‡	% change
1	Intact, pseudopregnant 6 days		7	88.8 ± 4.8§	46.1 ± 4.7	−48
2	Pseudopregnant 6 days + hypex.	1 wk	8	—	101.9 ± 9.0	—
3	*Idem*	1 "	9	81.4 ± 5.6	88.6 ± 7.8	+ 8.8
4	"	4 "	7	78.7 ± 4.7	76.3 ± 4.6	− 3.0
5	"	5 "	7	87.0 ± 4.0	90.1 ± 5.9	+ 3.6
6	"	5 "	6	—	83.7 ± 3.9	—
7	Pseudopregnant 6 days + autografts	1 "	7	83.3 ± 4.2	31.4 ± 5.1	−62
8	*Idem*	4 "	7	61.0 ± 4.3	30.1 ± 3.0	−51
9	"	5 "	9	—	30.9 ± 3.0	—

* All animals were made pseudopregnant by inj. of PMS and HCG.
† Left ovary was removed surgically under ether anesthesia just prior to inj. of 3.2 μg LH/100 g body wt via tail vein.
‡ Right ovary was removed 4 hr after LH inj.
§ Stand. error.

contrast, a significant decrease in ovarian weight following hypophysectomy. Less pronounced differences are seen in concentration of ovarian ascorbic acid in the various groups. Statistically, the only significant decrease in ovarian ascorbic acid level ($P = <.001$) occurred in those animals (Table I, group 8) which had pituitary autografts for 4 weeks.

A dramatic difference in responsiveness of ovarian ascorbic acid to LH is seen when the effect of hypophysectomy is compared to pituitary autografting (Table II). When given to animals hypophysectomized for periods of from one to 5 weeks LH was without significant effect on ovarian ascorbic acid (Table II, groups 2-6). The same dosage of LH given to animals bearing pituitary autografts led to depletion of ovarian ascorbic acid even greater than that obtained in intact, pseudopregnant rats (Table II, groups 7-9). The depletion seen in the intact and autograft bearing rats is, in all cases, highly significant ($P = <.001$).

Discussion. It has long been known that corpora lutea will remain in ovaries of adult rats for long periods after hypophysectomy (9). Such corpora lutea are physiologically inactive, however, in contrast to the functional corpora lutea which can be maintained for months in the adult rat by pituitary autografts(5). We have found that pituitary autografts in immature animals as used in the present study are incapable of maintaining ovarian weight for periods longer than 4 weeks (unpublished observations). Maintenance of ovarian weight is remarkably good for 4 weeks after operation although a significant decline in ovarian ascorbic acid level is already apparent (Table I).

The difference in response of ovarian ascorbic acid to LH in hypophysectomized animals as compared to intact or autograft-bearing rats is an important one. It indicates that, for LH to deplete ovarian ascorbic acid, the ovary must be under the influence of luteotrophin. This explains the rapid decline in ovarian ascorbic acid and the decrease in its sensitivity to LH after hypophysectomy as reported by McCann *et al.*(4). We also have found that a significant fall in concentration of ovarian ascorbic acid takes place during the first 24 hours after hypophysectomy. A return to a value not different from the level in the intact, pseudopregnant animal then occurs. It now seems likely that this initial decrease which follows hypophysectomy may be due to a release of LH from the pituitary during its removal. Whether or not this is the case, it is now obvious that hypophysectomized animals are basically unsuitable for assay of LH by the ascorbic acid depletion method. On the other hand, animals made pseudopregnant in the usual manner and subjected to the pituitary autografting procedure should prove useful in several

ways. Since such grafts appear incapable of secreting detectable amounts of either FSH or LH the problem of possible interference by endogenous LH in ovarian ascorbic acid depletion is removed. Likewise, in these animals the possibility of ovarian ascorbic acid depletion being due in part to LH-releasing factors as opposed to LH *per se* is eliminated. For these reasons, it would seem desirable to utilize such animals in conjunction with the usual assay animal in testing various agents and biological materials for LH-releasing substances. In this connection there is disagreement in the literature as to whether vasopressin and adrenaline will affect ovarian ascorbic acid in the hypophysectomized animal(2,4). Finally, it should be emphasized that for routine assay of LH we consider the procedure described by Parlow(1) using intact, pseudopregnant animals to be quite satisfactory. It will of course, be necessary to use intact animals as test objects in further search for LH-releasing substances.

Summary. Immature female rats were made pseudopregnant in the manner prescribed for the Parlow LH assay. Hypophysectomy of such animals on the sixth day of pseudopregnancy caused a gradual decline in ovarian weight over a 5-week period but concentration of ovarian ascorbic acid was well maintained. At periods of a week or more after hypophysectomy intravenous injection of LH produced no decline in ovarian ascorbic acid. In other animals the pituitary gland was autografted to the renal capsule on sixth day of pseudopregnancy. Ovarian weight was well maintained in these animals for 4 weeks although at 4 weeks there was a significant decrease in level of ovarian ascorbic acid. Intravenous injection of LH in pituitary graft-bearing rats led to depletion of ovarian ascorbic acid even greater than that found in intact, pseudopregnant rats. It is suggested that animals bearing pituitary autografts should prove valuable in the search for LH releasing factors.

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Studies of Serum Protein Abnormalities in Kala Azar.* (26340)

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Patients with kala azar have long been known to have hyperglobulinemia. Recently, moving boundary and filter paper electrophoresis of serum proteins from such patients have shown a nonspecific diffuse hypergammaglobulinemia and hypoalbuminemia without significant alterations in alpha or beta globulins(1,2,3,4,5). A few workers, however, have reported what they believed to be specific abnormalities. Benhamou *et al.*(6)

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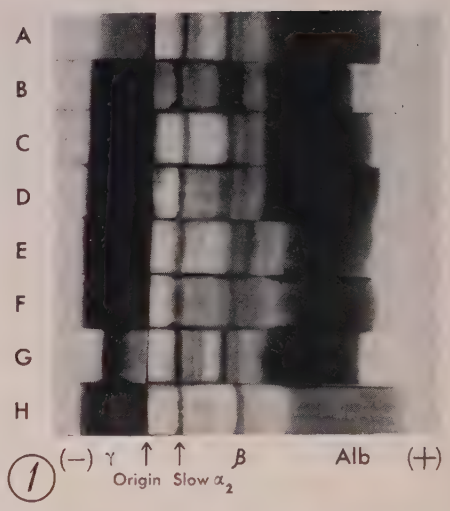
noted the presence of an "X fraction" with a more rapid mobility than albumin, as well as a gamma globulin of slower mobility than that of normal gamma globulin, the latter also identified by Cooper *et al.*(7). Sen Gupta(8) also found this slow moving gamma globulin, and emphasized that it is not seen in several other conditions characterized by hypergammaglobulinemia. Because of these conflicting reports and because the demonstration of a specific protein abnormality in kala azar may bear a relation to other dysproteinemias, the present studies were performed using starch gel and filter paper electrophoresis, immunoelectrophoresis, and the Ouchterlony gel diffusion technic.

Method. Sera from 5 patients with a diagnosis of kala azar established by bone marrow aspiration were obtained in Ceará, Brazil, a region where this disease is endemic. The sera were frozen and shipped to New York for study.

Total protein, albumin, and globulin were determined by a modified Kingsley method (9). Smithies' method was used for starch gel electrophoresis(10). Soluble potato starch was obtained from the Connought Laboratories. Gels were prepared at a concentration of 12.2 g % in 0.021 M borate buffer at pH 9.05. Electrophoresis was performed for 16 hours at 10°C with a potential of 4.5 volts per cm using a 0.30 M borate buffer. Filter paper electrophoresis was performed with the Spinco apparatus using Veronal buffer, pH 8.6, ionic strength 0.05. The paper and the gel strips were stained with naphthalene black B 200. Gel diffusion technics were performed by the Ouchterlony method as described previously by Korngold and Lipari(11).

Scheidegger's procedure(12) for micro immuno-electrophoresis was modified as follows: lantern slide covers were coated with a thin layer of Ion Agar No. 2§ at 1.2% in phosphate buffer (ionic strength 0.05, pH 7.6). The same buffer at ionic strength 0.1 was placed in the electrode vessels of the Spinco model R paper electrophoresis apparatus. After the antigen solutions were applied to the agar with a micro burette, the plate was inverted and the edges of agar on the plate were pressed against the filter paper wicks to assure good contact between them(13). A constant current of 45 mA was applied for 2 hours, during which the albumin migrated 12 mm from the origin. At the end of the run, 0.1 ml of an anti-human serum‖ was introduced into the troughs. Shadowgraphs were made 24 hours later.



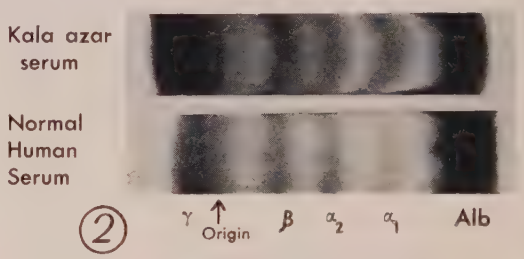


FIG. 1. Starch gel electrophoresis patterns of serum proteins. The origin is marked. A, normal; B, C, D, E, F, kala azar; G, multiple myeloma; and H, cirrhosis of liver.

FIG. 2. Filter paper electrophoresis patterns of serum proteins. The origin is marked. Upper pattern is from patient with kala azar and lower pattern is normal control serum.

Results. All serum globulin values were elevated. Analysis of each of the 5 sera by starch gel electrophoresis revealed a marked hypergammaglobulinemia (Fig. 1). There

§ Consolidated Laboratories, Inc., Chicago Heights, Ill.

‖ This antiserum, produced by immunizing goats with human serum fractions, was kindly supplied by Dr. R. T. Fisk, Hyland Laboratories.

was a concentration of protein in the slow gamma region of all 5 samples. In 3 of these samples, there was an additional protein concentration in the fast gamma region. Thus, one or 2 moderately well-defined, deeply-stained bands appeared in all of the samples. These bands were not as distinct as those commonly seen in multiple myeloma, but they were considerably more pronounced than those found in other forms of hypergammaglobulinemia, such as Laennec's cirrhosis. The remaining area of gamma globulin distribution appeared essentially normal. Variations in other components of the serum protein pattern after starch gel electrophoresis were related to differing haptoglobin types. One sample, B, showed a markedly elevated slow alpha-2 component.

Filter paper electrophoresis showed a diffuse elevation of the gamma globulin without any discrete narrow bands (Fig. 2). In 3 of the samples, a small amount of protein remained at the origin. These were the same 3 sera in which the fast-moving gamma component was observed after starch gel electrophoresis.

The Ouchterlony gel diffusion study revealed a reaction of identity when the 2 antigens, kala azar serum and normal serum, were compared using rabbit antisera prepared against Cohn fraction II (gamma globulin) as antibody. This indicated that the gamma globulins were not of the type seen in multiple myeloma(14). Each kala azar serum, however, contained increased amounts of macroglobulins.

Immuno-electrophoresis of the sera (Fig. 3) also showed a marked hypergammaglobulinemia, which is apparent from the diffuse nature of the zone of precipitate produced by the gamma globulin and by the fact that these zones moved to the antiserum troughs. Moreover, the gamma-1 macroglobulin was markedly elevated when compared with the normal sera run simultaneously, a finding which is in agreement with the results obtained by the Ouchterlony method. Only one serum, C, had an elevated beta-2A globulin.

Discussion. Our studies employing filter paper electrophoresis suggest that no specific

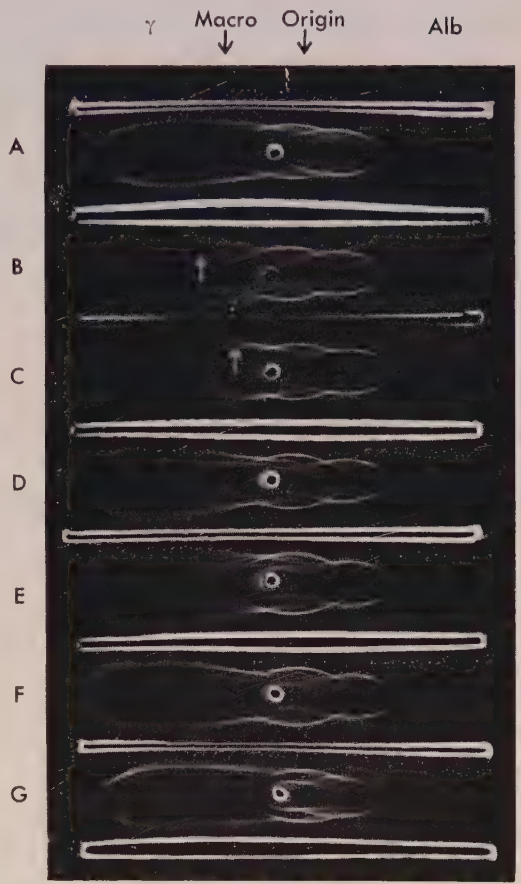


FIG. 3. Strips A and G contain normal sera; patients' sera are in the other strips. Gamma-1 macroglobulin is indicated by an arrow in strip B. The same band can also be seen in strips C, D, E, and F. Beta-2A globulin is indicated by an arrow in strip C which is the only one which shows this band.

protein abnormalities are demonstrable in the sera of patients with kala azar. The nonspecificity of the electrophoretic pattern on filter paper thus coincides with the majority of the previous results reported with this technic in this disease. The band remaining at the origin in 3 of the specimens probably represents fibrinogen or denatured protein rather than any specific protein alteration.

On the other hand, the definite protein concentration in the slow gamma region apparent in all the starch gel patterns differs from that seen either in multiple myeloma or in other conditions associated with hypergammaglobulinemia, such as cirrhosis and sarcoidosis. Simply stated, slow gamma

peaks in the patterns of the sera from patients with kala azar were more diffuse than those in multiple myeloma, and more discrete than those in other hypergammaglobulinemias. The 3 bands in the fast gamma region were found in the 3 samples showing the concentration at the origin on filter paper electrophoresis, and probably represent the same protein. Analysis of the filter paper and starch gel patterns revealed nothing that could be interpreted as the "X fraction" described by Benhamou.

We believe that the band in the slow gamma region reflects a protein abnormality in kala azar not found in several other pathologic conditions also characterized by hypergammaglobulinemia. It is conceivable that these bands represent a particular antibody response to one or another antigen derived from *L. donovani*. We should like to emphasize that with the technics used in this study, it is not possible to state whether the protein abnormality results from an increase of normal gamma globulins or from the appearance of abnormal proteins.

The observation that in kala azar a precipitation occurs when serum is added to water (Sia water test), together with the finding of cryoglobulinemia(15,16) suggested that increased concentrations of macroglobulins might also be present. This was found to be so in our study. Analysis in the ultracentrifuge was not performed to verify this, since results with this technic are generally unsatisfactory when frozen sera are used. Moreover, gel diffusion and immuno-electrophoresis are equally satisfactory for the detection of elevated macroglobulins.

Summary. Study of sera from 5 patients with kala azar revealed an abnormality in the starch gel electrophoretic pattern not seen in other diseases associated with generalized hypergammaglobulinemia. This abnormality consisted of a definite protein concentration in the region of slow gamma globulin. An elevation of macroglobulins was also found.

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Thiocyanate Secretion in Sweat in Cystic Fibrosis.* (26341)

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Cystic fibrosis is a disease of various exocrine glands, particularly the pancreas, bronchial mucus glands, and sweat glands. Sodium and chloride concentrations in the sweat in cystic fibrosis are approximately 5 times as high as in normal individuals(1).

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TABLE I. Sweat and Serum Thiocyanate.

Cystic fibrosis patients				Control subjects			
Patient	Sweat (mg %)	Serum (mg %)	Sweat / Serum	Patient	Sweat (mg %)	Serum (mg %)	Sweat / Serum
B.B.	3.7	5.3	.68	J.B.	.2	4.0	.05
B.B.	1.6	5.0	.32	D.B.	.2	4.3	.05
P.B.	.7	3.0	.23	J.C.	.6	6.5	.09
B.G.	3.4	4.7	.72	A.C.	.8	6.6	.12
M.A.	3.5	7.5	.46	D.C.	1.4	7.0	.20
R.B.	1.9	8.8	.22	C.A.	.6	7.0	.09
L.B.	2.4	9.9	.24	B.M.	1.2	6.5	.19
D.L.	1.4	7.9	.18	J.Z.	.7	6.8	.11
B.G.	3.0	8.8	.34				
Mean	2.40	6.77	.377		.713	6.08	.110

The defect appears similarly with respect to iodide as shown by sweat collection following ingestion of radioactive iodide(2). Only a slight (50%) elevation occurs in potassium (1,3). Unpublished data indicate that there is little or no elevation of sweat calcium in cystic fibrosis.

The present study concerns observations with thiocyanate, which has been considered to act as an halide physiologically.

Method. The subjects were given a 4% solution of sodium thiocyanate USP, in a dosage of 10 ml per square meter body surface, as estimated from height and weight. Two hours later, sweating was induced by wrapping the subjects in plastic sheeting, wool blankets and one or two 30-Watt electric heating pads for 20 to 30 minutes. The sweat was collected from the chest or upper back in 2 x 2″ gauze squares opened to size 4 x 6″. The sweat was then removed from the gauze by centrifugation with use of plastic golf tees to hold up the gauze in the centrifuge tubes. At the end of the sweating period, venous blood was drawn and allowed to clot. Colorimetric determinations of thiocyanate were then made in sweat and serum, by addition of ferric nitrate to a portion of the supernatant solution following deproteinization with trichloracetic acid(4).

Results. Serum and sweat concentrations of thiocyanate in infants and children are shown in Table I. The 2 tests on patient B.B. in the cystic fibrosis group were done 3 days apart. The 2 tests on B.G. were done 3 years apart.

The mean sweat/serum thiocyanate ratio for the cystic fibrosis patients is 0.377 ± 0.229.† The mean for control patients is 0.110 ± 0.056. The difference between the means (0.267) is 3.4 times the standard error of the difference between the 2 means (0.079).

The cystic fibrosis patients had sweat chloride levels ranging from 68 to 195 meq/liter with a mean of 122, as compared with the usual non-cystic fibrosis levels of approximately 20 meq/liter.

Summary. The mean sweat/serum ratio of thiocyanate in cystic fibrosis was 3.4 times the ratio in controls. This indicates that like sodium chloride and iodide, thiocyanate is involved in the defect of sweat electrolyte secretion in cystic fibrosis.

† Standard deviation.

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Effect of Ingested Thermally Oxidized Corn Oil on Fat Composition in the Rat. (26342)

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Previous studies(1,2,3) have shown that rats fed diets which contained oxidized oils did not grow as well and had larger or heavier livers than those fed fresh oils. Although thermally oxidized corn oil can be hydrolysed by pancreatic lipase *in vitro*(4), its rate of absorption from the intestinal tract was significantly less than that of fresh oil. The present study is concerned with the effects of hydrogenation, and of dilution of oxidized with fresh corn oil on absorption, growth, liver weight, and carcass and fecal fat composition.

Methods. A basal diet(5) which contained 12% of fat was used in all experiments. The test fat provided 10% and fresh cottonseed oil 2% of the basal diet. The diets were stored under nitrogen and refrigerated to minimize autoxidation of fat. Groups of 5 male weanling rats each were kept for 59 days in single cages, weighed on alternate days, and arbitrarily restricted to the same amount of food intake as those fed a basal diet containing 12% of a light-colored, refined corn oil. Samples of the feces were collected and frozen. After the test period the animals were sacrificed, the livers removed and weighed, and the carcass frozen and stored at –20°C until required for analysis. The carcass and fecal fats were extracted as previously described(6).

Aliquots of the fat samples were converted to their corresponding methyl esters prior to gas liquid chromatographic analysis. The procedure adopted was carried out as follows: Approximately 1 g of the fat to be esterified, 25 to 30 ml of methanol (anhydrous), and 50 to 100 mg of dry sodium methoxide were added to a small flask, refluxed for 45 minutes, poured into a separatory funnel, and diluted with 100 ml of water. The methyl esters were removed by extraction with petroleum ether (Skellysolve F), washed with water, and dried over sodium sulfate. Solvent was removed under vacuum on a rotary evaporator. Duplicate samples of methyl esters were prepared from all fats and stored under nitrogen in a refrigerator until analyzed. Wijs iodine values(7) and acetyl values(8) were determined in duplicate. The samples of oxidized corn oil, hydrogenated oxidized corn oil, and fatty acids of oxidized corn oil were prepared as previously described(9). The percentage of hydroxylated material in the dietary fats varied from 0 to 36.6%. This hydroxylated material was essentially a high molecular weight, polymeric material produced during thermal oxidation of corn oil. The remaining component acids are tabulated in Table I.

Gas chromatographic analyses of the methyl esters were carried out on an instrument constructed in our laboratory, which employs a thermal conductivity detector system. A 4 foot, ¼ inch internal diameter copper column was packed with 60-80 mesh Chromasorb impregnated with 30% by weight of succinic anhydride diethylene glycol polyester. The general method for preparing this type of column has been published(10).

Analyses were usually run at 190-205°C with a helium pressure of 11 lb per square inch, which resulted in a flow rate of about 60 ml of gas per minute. For a typical analysis of fatty acid methyl esters, 1-5 μl of the ester was injected into the column with a micro-syringe. The analysis required from 30 to 60 minutes to complete, depending upon the operating column temperature selected. Identification was based on comparison of retention time of the esters with those of authentic samples. The composition of each mixture analyzed was calculated on the basis of the area under each peak, as found by the triangulation method.

Results and discussion. Rats fed 10% of the diet as hydrogenated oxidized oil exhib-

TABLE I. Comparison of Dietary Supplement Fatty Acid Composition.

Fatty acid component	Fresh corn oil	Oxidized corn oil	Hydrogenated oxidized corn oil	Fatty acids from oxidized corn oil	10% oxidized fatty acids + 90% fresh fatty acids from corn oil
Linoleic	54.4	14.6	—	14.6	50.5
Oleic	28.8	28.3	.5	28.3	28.7
Stearic	2.3	3.6	46.5	3.6	2.5
Palmitic	13.0	16.4	16.4	16.4	13.3
Myristic	.2	—	—	—	.2
Lauric	.4	—	—	—	.3
Hydroxy acids	—	36.6*	36.6	36.6	3.6
Iodine value	126.0	71.0	55.0	71.0	120.0

* Determined as percentage of hexane-insoluble fatty acids.

ited less growth depression and less liver enlargement than those fed the non-hydrogenated material (Table II). The feeding of 10% of oxidized corn oil resulted in a statistically significant increase in liver weight and a decrease in weight gain when compared to the effects of feeding fresh corn oil. No significant differences in either liver weight or weight gain were observed when the animals were fed a mixture containing 10% of the fatty acids from oxidized and 90% from fresh corn oil. The effect of addition of such a small amount of oxidized material may not have become apparent in the short experimental period.

When oxidized corn oil which contained approximately 36% of polymeric hydroxylated material, the corresponding fatty acids, or the corresponding oil hydrogenated to an iodine value of 55, were fed to rats at the 10% level, a distribution of the component fatty acids essentially identical to that found in animals fed 12-hydroxy stearic acid, ricinoleic acid or triricinolein(11) was found in the carcass and fecal fat (Table III). From 4.1 to 7% of hydroxy acids seemed to be deposited in the carcass fat; again very similar levels to those observed when hydroxy acids were fed to rats(11). Ricinoleic acid fed at the 10% level resulted in increased liver weights and decreased growth, although digestibility of the ricinoleic acid was 85.8% (11) and that of ingested oxidized oils varied from 77 to 86%. Ingestion of a mixture of 90% fresh oil and 10% oxidized oil produced no significant changes in fecal or carcass fatty acid composition of the rat when compared to fresh corn oil. The observation that a significant quantity of hydroxy acids is deposited when rats are fed thermally oxidized corn oil is important since it demonstrates conclusively that hydroxylated compounds present in heated fats are absorbed when fed to animals.

Summary. Five groups of weanling rats were kept for 59 days in individual cages and fed adequate diets which contained 2% cottonseed oil and 10% of the following fats: 1) corn oil, 2) oxidized corn oil, 3) hydrogenated oxidized corn oil, 4) fatty acids from

TABLE II. Comparison of Biological Response of Rat to Ingested Fats.

Diet supplement	Changes in wt (g) in 59 days	Digestibility (%)‡	Percentage of liver/body wt
Fresh corn oil	204.2 ±20.3†	100.0	3.54 ± .41
Oxidized corn oil	157.0 ± 2.5*	77.2*	4.25 ± .20*
Hydrogenated oxidized corn oil	169.5 ± .9*	86.3*	4.12 ± .30*
Fatty acids from oxidized corn oil	162.5 ±14.1*	78.8*	4.68 ± .34*
10% oxidized corn oil + 90% fresh corn oil fatty acids	196.7 ± 7.1	96.1	3.13 ± .47

* Statistically significantly different from fresh corn oil group at 95% level.
† Stand. error of mean.
‡ (Wt gain on test fat)/(Wt gain on control fat, fresh corn oil) × 100.

TABLE III. The Influence of Dietary Fat on the Carcass and Feces Fatty Acid Composition of the Rat.

Fatty acid component	Fresh corn oil		Oxidized corn oil		Dietary supplements				10% oxidized corn oil fatty acids + 90% fresh corn oil fatty acids	
					Fatty acids from oxidized corn oil		Hydrogenated oxidized corn oil			
	Carcass	Feces	Carcass	Feces	Carcass	Feces	Carcass	Feces	Carcass	Feces
Linolenic	.26 ± .55†	—	.46 ± .55	0	.0 ± .0	—	.0 ± .0	0	.00 ± .0	0
Linoleic	36.10 ± 2.27	17.9	15.20 ± 2.88	29.5	23.72 ± 4.07	39.8	10.28 ± 1.41	5.4	29.78 ± 4.50	18.2
Oleic	31.96 ± 1.33	34.3	44.68 ± 2.91	23.1	44.50 ± 1.41	20.2	46.25 ± 1.59	21.9	33.78 ± 1.84	33.6
Stearic	2.89 ± .83	17.8	2.59 ± .76	29.9	3.43 ± 3.41	—	4.34 ± 1.73	33.6	2.64 ± .54	8.5
Hexadecenoic	5.07 ± .02	8.8	10.08 ± .25	—	9.23 ± 2.87	—	10.47 ± 3.03	—	5.67 ± 1.45	2.5
Palmitic	21.42 ± 2.42	25.6	23.14 ± 3.30	35.7	26.33 ± 2.84	33.1	24.48 ± 1.11	37.0	24.12 ± .79	13.8
Myristic	1.61 ± .67	10.2	2.74 ± .20	1.1	2.76 ± .56	1.9	2.48 ± .71	1.9	1.95 ± .30	.8
Tetradecenoic	.10 ± 1.04	—	.87 ± .54	—	.47 ± .00	—	.53 ± .51	—	.14 ± .52	—
Lauric	.27 ± .48	2.8	.34 ± .53	1.1	.52 ± .00	5.4	.42 ± .35	.4	.45 ± .34	.5
Hydroxy acids	1.50 ± 1.04	2.50*	6.98 ± 2.66	1.93*	4.18 ± 1.12	2.84*	4.27 ± 3.01	2.59*	1.88 ± 1.95	3.86*
Iodine values	99.68 ± 3.70	103.0	77.88 ± 2.7	53.8	79.74 ± 4.83	74.9	72.57 ± 1.52	44.2	90.02 ± 4.18	89.5

* Reported as percent hydroxyl for feces fats only.
† Stand. error of mean.

oxidized corn oil, and 5) 10% oxidized fatty acids plus 90% fresh fatty acids from corn oil. All animals were restricted to the same amount of daily food intake as those fed corn oil and samples of feces collected for lipid analysis. At the end of the test period the animals were sacrificed; the carcass lipids were extracted, converted to methyl esters and subjected to gas liquid chromatographic analysis. The results indicated that hydroxy acids, originating from oxidized fats, are deposited and influence the character of the normal mixed fatty acid composition of the carcass fat.

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Paper Electrophoretic Patterns of Human Serum Proteins Compared with Those of Lower Forms.* (26343)

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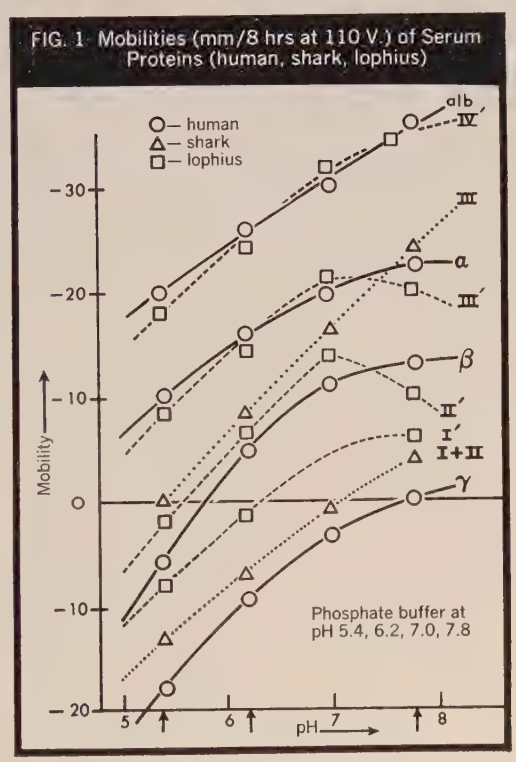
Although the patterns of sera from mammals have a general resemblance in number of components and their electrophoretic properties(1,2), this apparently does not apply or include(3,4,5,6,7) those from lower vertebrates (Elasmobranchi, Teleosts, Amphibia, Reptilia, Aves). Proteins with electrophoretic properties similar to human serum albumen were not observed in the shark(3,4,8) and in more than one genus and family of turtles(4,5,7). In this paper comparative observations were extended and include patterns from widely divergent forms. With each run of a given species, samples of human serum were included which were always taken from the same individual. Volumes of sera, time, ionic strength, voltage, amperage, paper strips, buffers, fixing and staining of proteins, and densitometer adjustments for recording were kept as uniform as possible. Each serum profile (Fig. 7) was prepared from average values taken from 3 or more individuals of a given species. From this study as summarized (Fig. 7) one can obtain qualitative information on relative concentration of proteins in 10 lambda samples from each of the various species. Number of components, their percentages, and migration can be compared with those of human serum.

Method and materials. Mobility studies with reference to pH levels were done on serum components of the shark (*S. acanthias*), goosefish (*L. piscatorius*) and human (Fig. 1). One tenth molar monobasic (KH_2PO_4) and dibasic (Na_2HPO_4) phosphates were combined in proportions to provide buffers at pH 5.4, 6.0, 7.0 and 7.8. Ten lambda samples from each of the 3 species were run simultaneously in a Durrum electrophoretic cell at the given pH levels. After 8 hours at 12 milliamperes and 110 volts, paper strips were removed; dried at 125°C for 30 minutes; stained with bromphenol blue for 6 hours; rinsed with 5% glacial acetic; dried at 125°C for 15 minutes and briefly exposed to ammonia vapor. The strips were then read with a self-recording densitometer. Distances in millimeters of the various components from point of application were recorded relative to pH (Fig. 1).

To provide serum patterns in which the electrically separable components were shown in higher contrast and in which relative proportions of each were readily recorded with the densitometer, 10 lambda serum samples were run with barbiturate buffer (pH 8.6, ionic strength 0.075) for 16 hours at 6 milliamperes and 110 volts. Paper electrophoretic strips were processed as mentioned above. A strip of human serum was included with each



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serum of a given species to be studied. Percentages and mobilities of components in an unknown serum were then compared with the gamma, beta, alpha and albumen components of the human serum as a frame (or grid) for points of reference (Figs. 2, 5, 7).

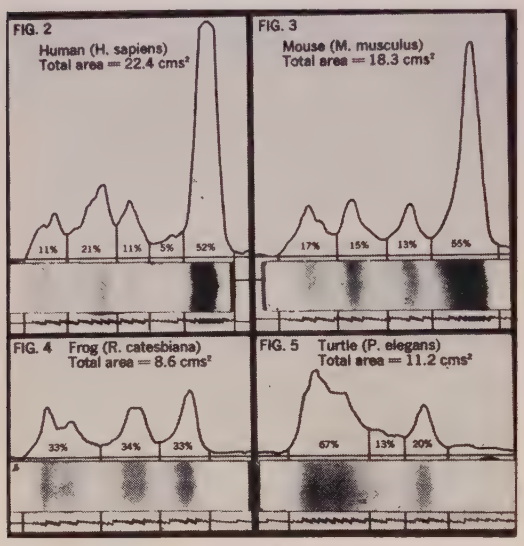


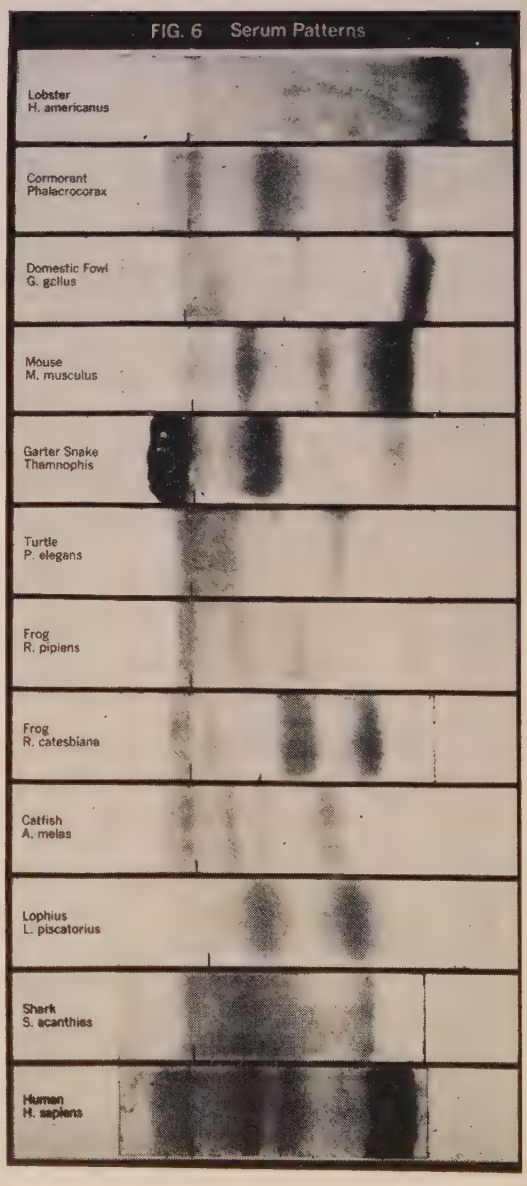
FIG. 2-5.

The components of each serum studied were numbered in sequence from the point of application toward anode as I to the final number of III to V.

Results. Mobilities of serum components of shark, goosefish and human were plotted in millimeters relative to pH 5.4, 6.2, 7.0 and 7.8 (Fig. 1). Four serum components of the human maintain their identities. Components I and II of shark serum as shown (Fig. 7), move at these pH levels as a single unit, and were found in proximity to human gamma. Shark (III) was found in relative positions to human alpha and beta. Four components of goosefish (Lophius) are found at these pH levels. Retention of electrophoretic properties of the components of the 3 sera at various levels of pH would indicate that each exists as a natural entity.

To prepare serum patterns in which the relative proportions of each component stand out in good contrast, 10 lambda samples of each serum were applied to paper strips and run in barbiturate buffer (pH 8.6) for 16 hours at 6 milliamperes and 110 volts. With each run of a given species, 2 or more strips of human serum were included. A comparison of the serum patterns follow.

Shark (*Squalus acanthias*). Blood samples were taken from tail veins of 18 sharks (6 ♂, 12 ♀), which had been taken from Frenchmen's Bay, Me., and supplied through the courtesy of Dr. Roy Forster. Three components were observed in most of the serum samples (Fig. 6, 7). Component II, 70% to 90% (Avg 82%) was located as compared with human serum in the position



of gamma, beta and alpha globulins (Fig. 7). Component III, 4% to 22% (Avg 16%) in a position relative to human albumen and alpha^2. In all patterns a small component I, 2%-4% (Av. 3%) was found within 15 millimeters from point of application toward the cathode (Fig. 2). Sera from 8 animals with recently ingested food (stomach contents) were turbid. Component III was as high as 10%-22% in these sera, and as low as 4%-8% in the others. Patterns from male and female were similar.

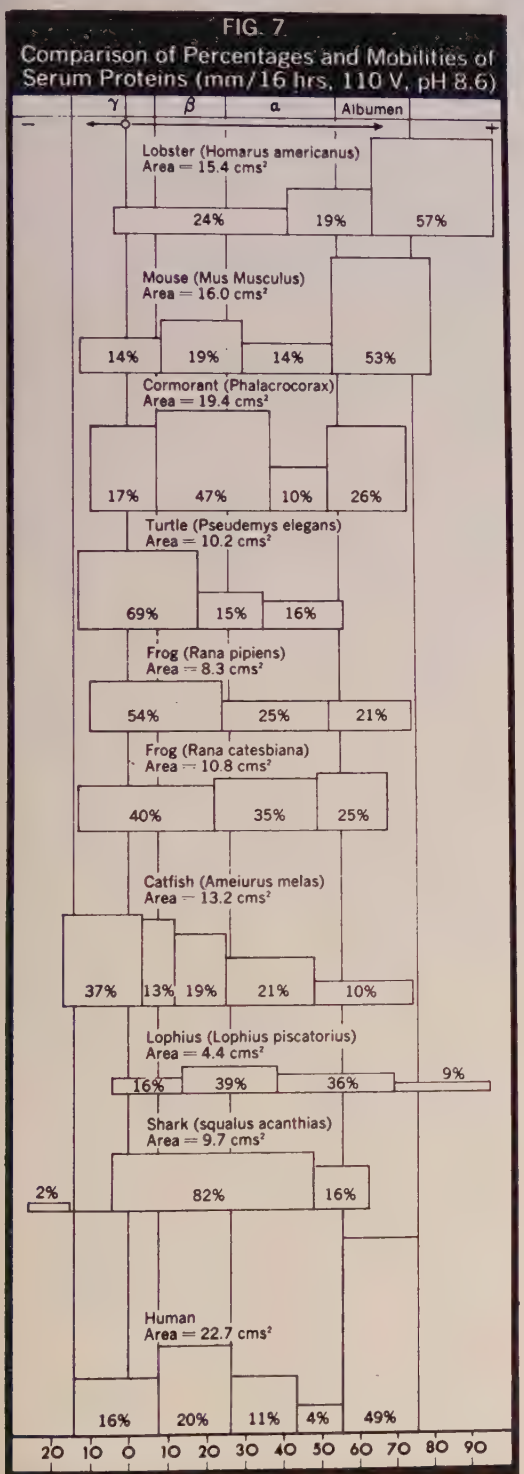


FIG. 7
Comparison of Percentages and Mobilities of Serum Proteins (mm/16 hrs, 110 V, pH 8.6)

The total stainable protein from 10 lambda samples of 18 animals as measured with the analytrol gave an average value of 9.7 cm^2 (Fig. 7). This value was approximately 43% of the total area of 22.7 cm^2 obtained from an equivalent volume of human serum. These values show the low level of protein in shark serum, and indicated that most of the proteins (78%-96%) had mobilities within the range of human globulins.

Goosefish (*Lophius piscatorius*). Blood samples were taken from renal veins of 8 animals. Four components were observed in the serum samples (Fig. 6, 7). The largest component (II) 39% to 44% (Av. 39%) had a mobility comparable to the range of beta and alpha^2 (Fig. 7). Component III, 28% to 41% (Avg. 36%) was found at levels of alpha^1 and albumen; I, 6% to 16% (Avg 13%) related to the range of gamma and beta; IV, 5% to 10% (Avg 9%) within and beyond the range of migration for human serum albumen. Total stainable protein of 10 lambda samples from 8 animals averaged 4.4 cm^2. This value was approximately 20% of the total area of 22.7 cm^2 obtained from an equivalent volume of human serum.

Catfish (*Ameiurus melas*). Eight catfish (3 ♀, 5 ♂) were used for obtaining serum from blood taken from tail veins. Five serum components were observed (Fig. 6, 7). The largest component (I, 33% to 40%) (Avg 37%), was within a range of mobility comparable to human gamma globulin; component II, 8%-14% (Avg 13%) within the range of gamma and beta; component III, 16%-22% (Avg 19%) was relative in migration to beta globulin; IV, 16% to 24% (Avg 21%) to the alpha complex; and V, 4%-12% (Avg 6%) to alpha^2 and albumen. Total

stainable protein of 10 lambda samples averaged 13.2 cm^2; 13.2/22.7 or 58% of amount obtained with the same volume of human serum.

Frog (*Rana catesbiana*). Blood samples were taken from the atria of 4 adult males. Three serum components were observed (Fig. 4, 6, 7). Component I was the largest; 32% to 47% (Avg 40%). Mobility was within the general level of human gamma and beta globulins. Component II, 32% to 40% (Avg 35%) was comparable in mobility to the alpha complex; III, 19% to 35% (Avg 25%) with a mobility within the levels of alpha[1] and extended into the range for human serum albumen. Total stainable protein of 10 lambda samples gave an average area of 10.8 cm^2; 10.8/22.7 or 48% of the amount obtained with an equal volume of human serum.

Frog (*Rana pipiens*). Blood was taken from the atria of 5 frogs and pooled. This procedure was done for each lot of 5 frogs in September, December and January. Total areas of stainable protein of 10 lambda samples in the same sequence were 17.7 cm^2, 11.3 cm^2 and 8.3 cm^2 respectively. This indicates that total serum protein decreases during the winter months. However, a comparison of the percentages of the 3 protein components in each of the 3 lots of pooled sera indicated that the relative proportions of the 3 components to each other remained approximately the same. Pooled sera of September frogs were used for comparative study (Fig. 6, 7). Component I was the largest, 46% to 56% (Avg 54%); with a mobility within the range of gamma and beta globulins. Component II, 20% to 30% (Avg 25%) within the mobility of the alpha complex; III, 15% to 25% (Avg 21%) was within the range for alpha[1] and albumen. Total stainable protein in 10 lambda samples of September frogs was 8.3 cm^2, or 8.3/22.7; 36% of an equivalent amount of human serum.

Turtle (*Pseudemys elegans*). Blood was taken from the atria of 3 males. Animals had been in laboratory tanks for 6 weeks without feeding. Three serum components were observed (Fig. 5, 6, 7). Component I was the largest; 59% to 76% (Avg 69%). The mobility of this component was at the level of human gamma and beta globulins. Component II, 10% to 26% (Avg 15%) was found within the levels of beta and alpha[2]; III, 14% to 18% (Avg 16%) a mobility within the range of the alpha complex. Total stainable protein of 10 lambda samples gave an average area of 10.2 cm^2; 10.2/22.7 or 45% of the amount obtained with human serum.

Cormorant (*Phalacrocorax*). Blood was taken from the wing veins of 3 animals and combined in one volume. Four serum components were observed. Component II was the largest (Fig. 6, 7), 47%, with a mobility within the range of human gamma and alpha[2] globulins. Component I, 17% was comparable to human gamma globulin in mobility; III, 10%, within the level of the alpha complex; IV, 26%, within the levels of human albumen. Total stainable protein in a pattern of 10 lambda of serum gave an average area of 19.4 cm^2; 19.4/22.7 or 81% of the amount obtained from an equivalent volume of human serum.

Mouse (*Mus musculus*). Blood samples from the atria of 3 Swiss mice were combined into one volume. Total of 6 volumes from 18 mice were used as sources of 6 serum samples. Four serum components were observed with percentages and mobilities comparable to human serum, except for the alpha which did not separate as 2 components (Fig. 3, 6, 7). Component IV was the largest, 49% to 56% (Avg 53%). Mobility of this component was similar to human albumen. Component I, 10% to 16% (Avg 14%) was similar in mobility to human gamma globulin; II, 16% to 24% (Avg 19%) similar to gamma globulin; III, 12% to 20% (Avg 14%) within the range for the human alpha complex. Total stainable protein in a 10 lambda sample gave an average area of 16.0 cm^2; 16.0/22.7 or 70% of the amount obtained with an equivalent amount of human serum.

Lobster (*Homarus americanus*). Lobster haemolymph was collected from the pericardal sinus of 8 specimens. Each was filtered and then applied as 10 lambda samples to electrophoretic paper strips, and processed

by the same methods as sera from vertebrates. Three components were observed which in their mobilities were not comparable to those of vertebrate sera (Fig. 6, 7). Component III, 55% to 60% (Avg 57%) was the largest component, with a mobility within and beyond the limits human albumen. Component I, 16%-30% (Avg 24%) was within levels of gamma, beta and alpha globulins. II, 17% to 25% (Avg 19%) within levels for alpha and albumen. Total stainable protein of 10 lambda samples averaged 15.4 cm^2; 15.4/22.7 or 68% of the equivalent value for human serum.

Discussion. Total stainable protein of 10 lambda serum samples from various species, as measured with a densitometer and recorded in cm^2, was highest for the human. Lower values were found for sera of lophius, shark, frog (*R. pipiens*) and turtle which were 20, 43, 36 and 45% respectively of human serum (Fig. 7). The frog (*R. catesbiana*), catfish (*A. melas*), lobster, mouse and cormorant were 48, 58, 68, 70 and 81% respectively.

Approximately half of the stainable protein of mouse or human serum was albumen (Fig. 2, 4, 6, 7). A similar albumen-like component was not found in the turtle (*P. elegans*). The serum component most proximal to the anode observed in patterns of shark (*S. acanthias*), lophius and frogs (*R. pipiens, R. catesbiana*) extended only over a portion of the range of mobility of human albumen (Fig. 7).

The globulin components of mouse serum patterns present a picture of general similarity in the relative proportions of each and in their mobilities to those of the human (Fig. 2, 4, 7). Serum pattern of mammalian species as reported in the literature have a remarkable resemblance(1,2). Although the serum patterns of cormorant and domestic fowl differ in relative proportions of globulins from those of the human (Fig. 6, 7), mobilities of gamma globulin and albumen approximated a similar range.

A component which moves some distance from the point of application toward the cathode was observed in serum patterns of turtle, frogs, catfish, lophius and shark (Fig. 7) and in this respect resembles human gamma globulin. However, the range of mobility and proportionate amounts present, differ so that they cannot be said to be comparable. A striking difference in the pattern of the garter snake is shown in Fig. 6. In this species the largest component is a fraction which has a mobility comparable to human gamma globulin. This observation was in agreement with that of Dessauer(9).

The serum pattern of lobster is in no way comparable to those of the vertebrates. It has a resemblance to the pattern of *Carcinus maenas* which was described as 3 components (10). The gamma globulin fraction as observed in mammals was not found in lobster serum.

Conclusions. The paper electrophoretic pattern of human serum has a general resemblance to other mammalian species in number of components and their electrophoretic properties. Globulin and albumin fractions of avian species (domestic fowl, cormorant) resemble in mobility those of the human. Serum components of lower vertebrates differed in their electrophoretic properties from those of the human and thus are not comparable. The serum pattern from an anthropod, *H. americanus,* was completely different from the patterns obtained from vertebrates.

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Comparison of Lipoprotein Lipase and Clotting Activity in Lymph and Plasma after Heparin.* (26344)

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A high concentration of lipoprotein lipase or "clearing factor" characteristically appears in blood following parenteral administration of heparin. The activity of this enzyme in other extracellular fluids has not been definitely established. Spitzer stated that clearing factor did not occur in thoracic duct lymph following intravenous heparin (1). Young and Freeman found that post-heparin lymph from 2 rabbits had lipolytic activity(2). Recently Dumont demonstrated that no clearing factor activity was present in the post-heparin lymph from 5 humans despite its presence in blood(3).

In the studies to be reported lipoprotein lipase was measured in thoracic duct lymph and blood plasma before and after heparin administration in 7 dogs and in one patient. In every experiment this enzyme was found in moderate to high concentrations in post-heparin lymph. Such lymph also had greatly prolonged clotting times.

Materials and methods. In the 7 dogs the thoracic duct was isolated in the neck and cannulated with polyethylene tubing.† Each dog had been given a meal of lard (300 g) and commercial dog food pellets 1-2 hours before the procedure. Anesthesia was maintained with sodium pentothal. Usually each animal received an intravenous infusion of 500 ml of 5% glucose in water during the experiment. Blood and lymph were collected into chilled glass tubes containing 1.85% potassium oxalate solution before and at intervals up to 280 minutes after injection of heparin. Heparin‡ was given intravenously in a dose of 150 units/kilo of body weight. In some experiments protamine§ in an equivalent dose (1.5 mg/kilo of body weight) was injected intravenously 60 minutes after administration of heparin. Enzymatic activity of the blood-plasma and lymph specimens was determined after incubation with coconut oil for 2 hours by methods described previously(4). Of the 2 methods used for quantitation of lipoprotein lipase in lymph, measurement of glycerol proved to be more reliable than reduction in optical density of the lymph-coconut oil mixture.

The one human studied was a young woman who developed chylothorax following repair of an interventricular septal defect. The lymph or chyle was obtained by thoracentesis‖ with as complete an aspiration as possible before and 5 hours after 5,000 units of heparin intravenously and 10,000 units subcutaneously.

Clotting times of blood and lymph were determined by the method of Lee and White (5). Lymph triglyceride was measured by the method of Van Handel and Zilversmit (6).

Results. Pre-heparin lymph had virtually no lipoprotein lipase. Lymph collected at intervals from 10 to 260 minutes after intravenous heparin always had lipoprotein lipase activity greatly increased over the zero or trace amounts found in pre-heparin samples. Fifty-two separate lymph specimens from 7 dogs were so tested. Table I shows typical results for lymph and blood before and after

TABLE I. Lipoprotein Lipase in Lymph and Blood before and after Heparin (Expressed in Terms of Glycerol Production μM/ml).

Dog	Pre-heparin		Post-heparin (20-30 min.)	
	Lymph	Blood	Lymph	Blood
1	.06	.08	.32	.82
2	.04	.02	.48	.86
3	0		.18	.70
4	.02	.04	.36	.60
5	.01		.40	.80
6	0	0	.47	.70
7	.02		.26	

* Supported by research grants from Nat. Inst. Health and Am. and Iowa Heart Assn.

† PE Clay-Adams.

‡ Sodium heparin (Abbott).

§ Protamine sulfate (Eli Lilly).

‖ Courtesy of Dr. J. L. Ehrenhaft.

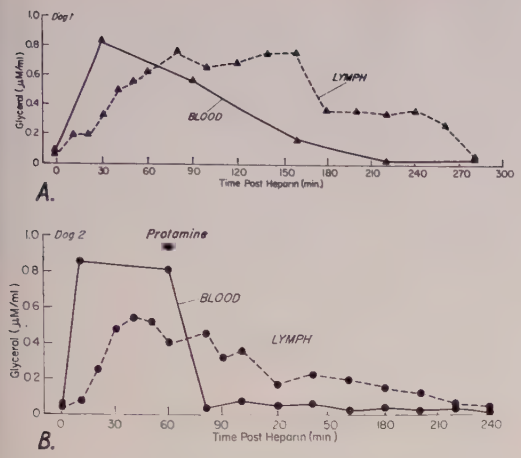

FIG. 1. A. Lipoprotein lipase in blood and lymph before and at intervals of time after intrav. heparin (Dog 1). B. Effect of protamine upon lipoprotein lipase in blood and lymph (Dog 2). Protamine was inj. at 70 min. post-heparin.

heparin. By the 20-30 minute period each dog had a large rise in enzymatic activity in lymph which was considerably lower than blood levels. In Fig. 1-A sequential data for both lymph and blood are given for Dog. 1. Initial values of lipoprotein lipase after heparin were much higher in blood than in lymph. At 80 minutes postheparin, however, concentration of the enzyme was greater in lymph than in blood. In fact the peak value of lipoprotein lipase in lymph (0.76 μM/ml of glycerol) was almost as high as the 30 minute post-heparin value for blood (0.82 μM/ml of glycerol). Lipoprotein lipase was elevated in lymph for 270 minutes. In blood, it was elevated for only 180 minutes. Intravenous injection of protamine 60 minutes after giving heparin seemed to accentuate this disparity between lymph and blood (Fig. 1-B).

The pre-heparin lymph obtained in the patient with chylothorax had little lipoprotein lipase (glycerol release of 0.02 μM/ml). Five hours after heparin, the lymph contained 5 times more lipoprotein lipase (glycerol 0.10 μM/ml). At this same time blood plasma produced 0.54 μM/ml of glycerol.

Clotting activity before and after heparin. Clotting times of lymph in 5 dogs varied from 3 to 15 minutes before heparin administration and increased to clotting times of over 60 minutes within 10 to 30 minutes after heparin. Blood clotting times in these same animals were 2-4 minutes before heparin and were elevated to over 60 minutes when measured 10-20 minutes after heparin. Lymph clotting time remained elevated after the whole blood clotting time had returned to pre-heparin values. In Dog 1, for example, lymph clotting time remained at 60+ minutes for 150 minutes post-heparin, whereas whole blood clotting time had declined to 15 minutes at 90 minutes post-heparin and was 3 minutes long at 150 minutes post-heparin.

Validity of methods to determine lipoprotein lipase. The data in Table II indicate that lipoprotein lipase cannot be measured by a change in optical density of the incubation mixture when the lymph sample is itself lipemic and has a high initial optical density. Little or no clearing occurred despite evidence of the enzyme as indicated by glycerol production. Table III further documents the inadequacy of the clearing method when the incubation mixture has an initial optical density above 1.0.

Discussion. These experiments provide evidence that lymph contained high concentrations of heparin and the enzyme lipopro-

TABLE II. Comparison of Two Methods for Lipoprotein Lipase Determination.

Dog	Post-heparin specimen	Optical density (units) of: Blood plasma or lymph	Incubation mixture Initial	Final	Change	Glycerol production (μM/ml)
1	Blood —40 min.	.02	.59	.20	.29	.82
	Lymph—30 "	1.80	1.90	1.90	0	.32
	" —80 "	1.25	1.25	1.17	.08	.66
4	" —40 "	1.20	1.25	1.20	.05	.40
	" —80 "	.69	.92	.76	.16	.46
6	Blood —30 "	.04	.57	.04	.53	.70
	Lymph—30 "	1.70	1.70	1.60	.10	.47

TABLE III. Analysis of Lipoprotein Lipase Determination.

Substrate*	O.D. of incubation mixture (units) Initial	Final	Change	Glycerol production (μM/ml)
Exp. 1				
Coconut oil 2.5%	.50	.15	.35	.94
" " 15.0%	1.29	1.26	.03	.98
Exp. 2				
Dog 1 chyle, diluted ⅓ with water	.65	.40	.25	.66
Dog 1 chyle,† undiluted	1.05	.96	.09	.68

* Incubation mixture for each experiment consisted of .12 ml of substrate and 2.5 ml of post-heparin plasma with an optical density of .06.
† Triglyceride content = 4220 mg %.

tein lipase after administration of intravenous heparin. Furthermore, lymph retained both anticoagulant and lipolytic activities for a considerably longer period of time than they could be demonstrated in the blood. Heparin is known to appear in the urine following intravenous injection(7). Its occurrence in lymph as indicated by a biological test of its presence (prolongation of clotting time) further supports the idea that it rapidly diffuses through the capillary endothelium. Thus the rapid decline in blood concentration of heparin given intravenously (7) is probably caused as much by its diffusion into the interstitial fluid compartment as by its inactivation or excretion. It is not known that this greater duration of heparin activity in the interstitial tissue fluid has clinical implications. Conceivably heparin may exert its anticoagulant or antilipemic effects for a longer period than might be indicated from tests of its presence in blood.

Lipoprotein lipase reached a maximal concentration much later in lymph than in blood. The slow transit time of lymph enters into the collection problem. In Dumont's study no lipoprotein lipase was demonstrated in human post-heparin lymph from the thoracic duct collected only 20 and 30 minutes after heparin(3). Since the transit time of lymph through the thoracic duct may take longer in man than in the dog(8), perhaps later samplings might have revealed the enzyme.

Summary. Lipoprotein lipase was found in the post-heparin thoracic duct lymph of the 7 dogs studied and in the post-heparin lymph aspirated from the chest in a patient with chylothorax. This enzyme reached a maximal concentration later and remained longer in lymph than in the blood. Post-heparin lymph also reflected the presence of heparin by greatly prolonged coagulation times. Evidence was given that lipoprotein lipase should be determined by measuring its lipolytic activity and not by "clearing" technics alone.

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Sex Difference in Lipid Content of Adrenal Glands in Mice. (26345)

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Sex differences in adrenal cortical sudanophilia(1) and in eosinophil counts(2) have been observed in mice. While dissecting adrenals of several inbred strains of mice it was noted that female adrenals appeared grossly larger, and more yellow and opaque than those of males which were somewhat translucent. A study was therefore undertaken to characterize further the adrenal cortical sex difference in lipids by histochemical and chemical means. Eosinophil counts were also done in an attempt to obtain simultaneous physiologic data.

Materials and methods. Eleven male and 11 female virgin 7 months old STR/N mice were maintained for 7 months after weaning on sawdust in plastic cages at 26.5 ± 1.0°C. They received Purina Laboratory chow with water *ad libitum*. Between 2 and 3 p. m. male and female mice were alternately sacrificed with ether and exsanguinated from the right ventricle of the heart with heparinized syringes. Eosinophil counts were made in a Levy counting chamber using the diluting fluid of Speirs(3). The adrenals were weighed individually on a 50 mg Roller-Smith precision balance. One adrenal of each animal was fixed in 10% formalin, frozen sections were made and one slide of each was stained with hematoxylin and eosin (H & E) and a second with oil red O(4).

The other adrenal was frozen immediately and set aside for tissue analysis. The protein was estimated following micro-Kjeldahl digestion and analysis for nitrogen with Vanselow's modification of Nessler's solution(5). Total lipid was determined by the potassium dichromate method as described by Bragdon (6). Phospholipid was estimated by determination of phosphorus by Fiske-Subbarow method as outlined by Umbreit, Burris and Stauffer(7). A modification of Zak's ferric chloride(8) method was used to determine cholesterol after hydrolysis and conversion to its digitonide.

Results. Plates I and II show typical female and male adrenal gland stained with oil red O. The zona fasciculata of the female mice is stained intensely with oil red O over its entire extent. In general, the zona fasciculata of the male stained less intensely. The inner half as well as columns and patches of fasciculata takes little or no stain. Individual cortical cells of the female fasciculata tend to have larger numbers and more intensely staining round droplets which fill the cytoplasm. The reticularis stains only in occasional clumps particularly in its inner zone in both males and females. Glomerulosa of both male and female mice are stained less intensely than fasciculata, but stained somewhat more heavily in females than in males. Sections stained with H & E reveal more cortical cytoplasmic pallor, which corresponds to lipid positive areas, in females than in males. Similar observations were made on several other inbred strains (BALB/c, C57/BL and DBA/2JN).

Results of the chemical analysis and eosinophil counts are shown in Table I. Adrenal weights for the female mice were greater than those of the males even though body weights of males were heavier than of females. Total lipid content of female adrenals was twice that of the male, while total lipid concentration was slightly though significantly higher in the females. Much larger cholesterol content and concentration were found in female adrenals than in the males. In the female adrenal, total cholesterol represented twice as much of total lipid than in the male. Phospholipid content, but not concentration, was significantly higher in females. Protein concentration was significantly lower in the female adrenal, by an amount proportional to the higher total lipid content.

Discussion. Higher lipid content in female

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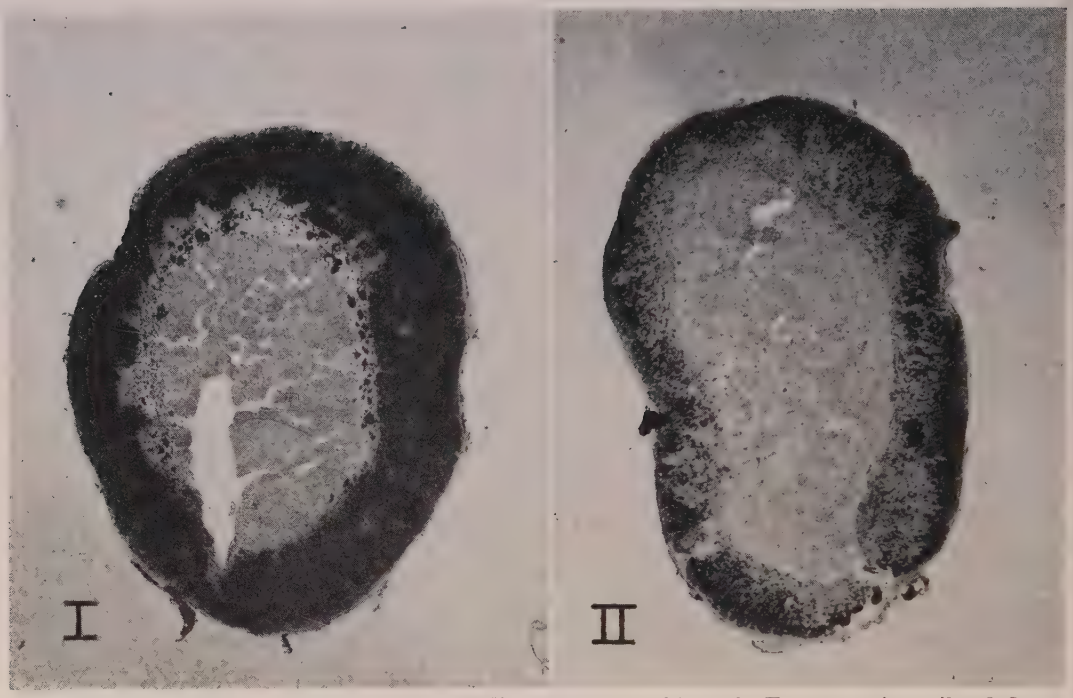

FIG. 1. Adrenal gland of female STR/N mouse, 7 mo old, ×30. Frozen section, oil red O stain. Note intense staining of majority of cortex with less staining of zona reticularis and glomerulosa.

FIG. 2. Adrenal gland of male STR/N mouse, 7 mo old, ×30. Frozen section, oil red O stain. There is less intense cortical staining than in female adrenal. Patchy areas of zona fasciculata are devoid of stain, particularly in inner portion.

mouse adrenal cortex, particularly in the zona fasciculata, have been observed using oil red O. Part of the differences in lipid content have been further shown in Table I to be due to cholesterol, a key precursor of the steroid hormones(9). Whitehead(1) showed that lipid as characterized by Sudan III staining was relatively more abundant in female adrenals cortex than in male at 150 days in mice, and that cholesterol staining (Schultz Reaction) followed the same pattern as the Sudan III stain(10). The protein and phospholipids, though their concentrations are lower in the female adrenals, are

TABLE I. Lipid and Protein of Adrenal Gland and Eosinophil Counts of 7 Months Old Male and Female STR/N Mice.

	Male	Female	p
Body wt, g	32.6 ± .66 *	23.1 ± .83	<.01
Adrenal wt, mg	4.16 ± .207	7.25 ± .260	<.01
mg/100 g body wt	12.7 ± .50	31.5 ± .99	<.01
Lipid, total, mg	1.08 ± .51	2.22 ± .127	<.01
mg/100 mg adrenal	26.3 ± 1.10	30.7 ± 1.47	<.05
Cholesterol, mg	.173± .132	.747± .459	<.01
mg/100 mg adrenal	4.19 ± .299	10.23 ± .547	<.01
mg/100 mg lipid	16.1 ± .92	34.4 ± 1.40	<.01
Phospholipid, mg	.128± .0093	.210± .166	<.01
mg/100 mg adrenal	3.09 ± .179	2.79 ± .250	>.05
Protein, mg	.88 ± .093	1.19 ± .064	<.02
mg/100 mg adrenal	21.5 ± 2.24	16.4 ± .84	<.05
Eosinophil count, cells/mm³	161 ±50.5	80.6 ±19.8	>.05

* Mean ± stand. error.

present in sufficient amounts in the total adrenals to imply greater amount of cellular material in the female adrenals.

Halberg(2) showed that eosinophil counts of female mice were lower at all times of the day than those of males. The present findings are in agreement with this observation. Adrenal cortical steroids are known to depress eosinophil counts(11). This suggests that steroid activity may be higher in female than in male mice. The finding of more cholesterol in the female mouse adrenal than in the male suggests that female mice may have higher stores of steroid precursors and possibly of steroids themselves in the cortex. The zona fasciculata is thought to elaborate glucocorticoids which are most effective in depressing circulating eosinophil levels(11). It is of interest that the sharpest differences between male and female adrenal glands exist in the zona fasciculata.

Summary. The zona fasciculata and to some extent the zona glomerulosa of the adrenals of female STR/N mouse stained more intensely and uniformly for lipid with oil red O than those of the males. The female adrenal was considerably larger than that of the male although body weights of the male were larger than the female. The absolute amounts of total lipid, cholesterol and phospholipid in the adrenals in female mice were higher than in males. Eosinophil count of female mice was lower, but not significantly so, than male counts.

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Antigenicity of Cholesterol Induced Chicken Atheroma.* (26346)

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These experiments were undertaken to determine whether or not the lipid or lipoprotein complex comprising the central mass of an experimentally produced arterial atheroma could stimulate specific antibody formation, and to clarify the serologic characteristics of such an antibody. It was also planned to investigate the *in vivo* effects of such anti-atheroma antibody on lesions of experimental atherosclerosis. Antigenic properties of experimental atheroma and normal intima were to be compared.

Methods and materials. The chicken was chosen as the experimental animal. The necessary dietary conditions for atheroma induction in the chicken have been well defined and are easily reproducible(1).

Eight-week-old HYLINE leghorn cockerels (Corn Belt Hatcheries, Joliet, Ill.) were

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placed on a diet of 2% cholesterol, 5% cottonseed oil and 93% chicken maintenance mash. When necessary, by addition of sucrose the protein content of the commercial mash was adjusted to 12-13%, a level optimal for atheroma production(2). The birds exhibited gross fatty lesions after 6-10 weeks and many plaques showed calcification at 16 weeks.

Antigens. Material from atheroma to be tested for antigenicity was obtained from birds which had been on the diet for between 8 and 16 weeks. Material from normal arterial vessels to be tested for antigenicity was obtained from 16 week or older leghorn cockerels which had been fed commercial chicken maintenance mash. The test material was removed from the thoracic aorta and brachiocephalic arteries.

Atheromatous material was scraped from diseased vessels with a razor blade and suspended in saline to a concentration of 0.3-0.4 mg protein/ml (micro-Kjeldahl analysis). Microscopic sections of scraped vessels showed that only atheromatous intima had been removed. Normal intima was removed by placing the intimal surface of normal vessels on a glass plate and scraping away the adventitia and most of the media. This antigen, therefore, contained some normal media in addition to normal intima and might more properly be called normal intima-media antigen, but for the sake of simplicity will be referred to as intima antigen. It was suspended in saline to a concentration of 0.8-1.0 mg of protein/ml. Both suspensions were prepared in a glass tissue homogenizer. All preparations were carried out at 0-5°C. Atheroma antigen was used the day of preparation and intima antigen was used up to one week following preparation.

Various normal chicken organs were prepared for use in studies on antibody specificity. The organ was homogenized in saline with a Waring® blender and the suspension centrifuged 10 min at 3000 rpm. The sediment was washed twice with saline and homogenized in a glass tissue grinder. This suspension was centrifuged 45 min at 30,000 rpm. The resulting sediment was resuspended to a concentration of approximately 1 mg of protein/ml when used for adsorption studies. The entire procedure was carried out at 0-5°C.

Immunization. Three adult, male albino rabbits were immunized with atheroma antigen and 3 similar rabbits were immunized with normal intima antigen. Approximately 0.1 g (wet wt) of antigen (representing the material from 2 or 3 chickens), suspended in 3 ml of Freund's adjuvant (Difco), was injected subcutaneously into the back of each animal. Booster injections were given after 3 weeks, 11 weeks, and 9 months. Rabbits were bled following the eleventh week and ninth month of booster injections and the sera stored at –20°C until used. Sera, prior to their use in complement fixation studies were heated at 56°C for 30 min.

Serology. Antisera were assayed by complement-fixation(3). In the presence of two-100% hemolytic units of guinea pig complement, 0.1 ml of a dilution of antigen was added to 0.2 ml of various dilutions of antiserum. Mixtures were incubated for 30 min at 37°C. Residual complement was detected by addition of 0.2 ml of a 2.5% suspension of amboceptor-sensitized sheep rbc. After incubation for 30 min at 37°C, the titer was recorded as the lowest serum dilution showing complete hemolysis. Appropriate anti-complementary controls were run with each experiment. To study the behavior of the antigen-antibody systems in the zones of antibody excess, equivalence and antigen excess, isofixation curves were derived by plotting the antiserum titer obtained during block titration with varying concentrations of antigen(4).

Results. Antibody response. Complement-fixing antibodies against homologous antigens were obtained in about equal titer following immunization with either atheromatous or normal intima. Antisera obtained at 9 months and used for these studies all had titers of 1/512. The activity of suspensions of both atheromatous and normal intima in complement-fixation was found primarily in the sediment following centrifugation at 30,000 rpm for 1 hr in a Spinco ultracentrifuge (#40 rotor).

Antibody characterization. Adsorption of

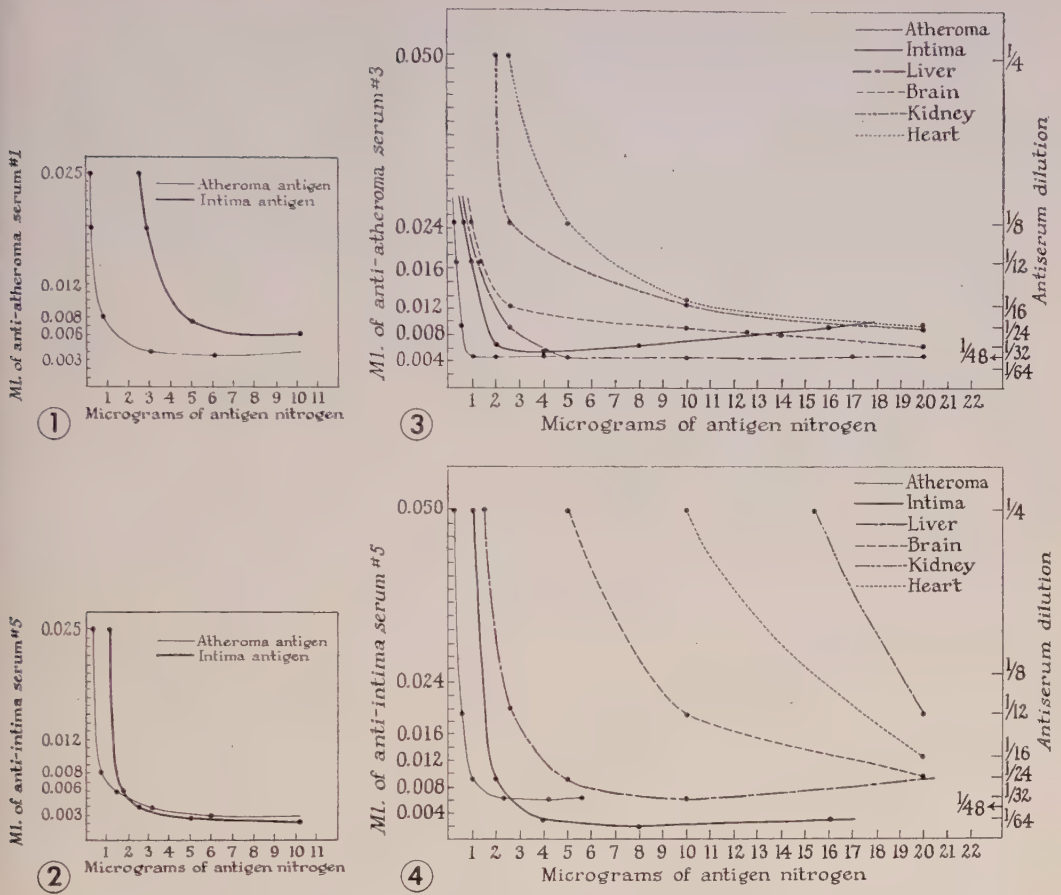

FIG. 1. Isofixation curves for an anti-atheroma serum *vs* atheroma antigen and normal intima antigen.

FIG. 2. Isofixation curves for an anti-intima serum *vs* intima antigen and atheroma antigen.

FIG. 3. Isofixation curves for an anti-atheroma serum *vs* various tissues.

FIG. 4. Isofixation curves for an anti-intima serum *vs* various tissues.

anti-atheroma or anti-intima rabbit sera with sheep or chicken erythrocytes or with guinea pig kidney suspensions resulted in marked reduction of antibody titer in complement fixation assays. All studies on specificity and cross-reactivity were therefore carried out with heterophile adsorbed antisera (equal volume of washed, packed sheep erythrocytes; room temperature; 45 min.; 2 adsorptions).

By means of isofixation curves a suggestion of antigenic specificity could be obtained with anti-atheroma but not anti-intima sera. The anti-atheroma sera exhibited higher titers when reacted with homologous atheroma antigen rather than intima antigen (Fig. 1). The reverse situation was not true, that is, anti-intima sera exhibited nearly identical titers when reacted with either atheroma or intima (Fig. 2). The suggested specificity of anti-atheroma serum was further supported by adsorbing both anti-atheroma and anti-intima sera with normal intima sediment. Following 2 adsorptions for 20 min at room temperature, the titer of the anti-intima serum was significantly reduced from 1/59 to 1/22 but the anti-atheroma serum titer remained constant at 1/29. Adsorption of antisera with atheroma suspension was technically impossible.

Both anti-atheroma and anti-intima sera were found to cross react with other tissues, particularly liver. To see if the antisera had specificity for their homologous antigens,

they were adsorbed twice with liver sediment, and the resulting titers *vs.* liver or homologous antigen were assayed.

Neither anti-atheroma nor anti-intima serum reacted with liver after the second adsorption. Two adsorptions led to a marked decrease (1/48 to 1/8) in titer of anti- atheroma serum *vs.* atheroma and a lesser decrease (1/64 to 1/32) in titer of anti-intima serum *vs.* intima.

These experiments indicated that anti-intima serum was made more specific for its homologous antigen by adsorption with liver, whereas anti-atheroma specificity was not increased. Adsorption studies with kidney antigen also showed anti-atheroma serum to lack specificity. To rule out non-specific adsorption by these organs, rabbit anti-diphtheria toxin serum was adsorbed similarly with liver and kidney. No change in titer was observed.

The reactivities of anti-atheroma and anti-intima sera with chicken atheroma, intima, liver, kidney, brain, and heart were further analyzed by means of isofixation curves (Fig. 3 and 4). Comparison of the two figures indicated that anti-atheroma serum cross-reacted with these organs to a greater degree than anti-intima serum. In the zone of antibody excess, anti-atheroma serum reacted moderately only with liver. These experiments therefore correlated in part with the previous adsorption studies suggesting anti-atheroma serum to have little tissue specificity.

In vivo studies. Three ml of anti-atheroma serum was injected intravenously into six 17-week-old atherosclerotic cockerels which had been on the atherogenic diet 7 weeks. Three of the animals were sacrificed after 5 days and the remainder after 8 days. A similar experiment using anti-intima serum and normal 17-week-old cockerels was performed. Organs were fixed in 10% formalin and sections of aorta, heart, liver, and kidney were examined for evidence of histologic change. No consistent histologic differences were observed in the tissues of the experimental and the 17-week-old noninjected controls.

Discussion. Since much of the rabbit anti-atheroma antibody was in response to a heterophile antigen, goats and guinea pigs were also immunized in a separate study (unpub). Both species had a prompt antibody response to chicken atheroma but no heterophile antibodies were detected. The decrease in titers of rabbit antisera following adsorption with Forssman positive tissues and lack of heterophile antibody response during immunization of Forssman positive animals indicated Forssman antigen is present in chicken atheroma and normal chicken intima. This reaffirms the known distribution of Forssman antigen(5,6).

The identity of substances responsible for antigenicity of chicken atheroma was not determined. Lipids such as cholesterol, cholesterol esters and phospholipids are among the principal chemical constituents of chicken atheroma. Many of the lipids may be free but some are probably in the form of protein complexes. The antigenicity of free lipids has not been demonstrated but ample evidence is available that lipids may function as haptenes and confer antigenic specificity when coupled to a suitable protein carrier (7,8). In addition to the possible lipid-protein antigens the cellular components of atheroma material may be antigenic.

A suggestion of anti-atheroma specificity in relation to normal intima was shown with rabbit antisera. Similar specificity was demonstrated with goat anti-atheroma sera but could not be shown with guinea pig anti-atheroma sera (unpub). Atheroma antigen is antigenically related to the stroma or parenchyma of other organs, particularly liver and brain. Lipoproteins may be the important common antigens since they are found in cell walls and in parenchymal cell nuclei of many organs(9). The marked cross-reactivity of anti-atheroma sera suggests the absence of antigens unique for chicken atheroma.

Lack of observable *in vivo* activity of antisera may be related to their titers, specificities and the poor opportunity for mechanical contact between injected antisera and aorta. Modification of the course of experi-

mental atherosclerosis by injection of anti-atheroma antibodies remains to be demonstrated.

Summary. Rabbit antibodies were produced in response to cholesterol induced chicken atheroma and chicken normal intima. Properties of these antisera were studied by complement fixation and described by isofixation curves. It was found that anti-atheroma sera reacted best with atheroma antigen and in addition reacted well with a wide variety of other chicken tissues. Anti-normal intima sera reacted well with either intima or atheroma antigen and had a limited spectrum of reactivity with other tissues. *In vivo* activity of these antisera could not be demonstrated.

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Effect of Potassium on Membrane Current of Single Ranvier Node During Excitation. (26347)

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When a frog nerve fiber is immersed in Ringer solution, containing 30-90 millimolar potassium chloride, and the resting membrane potential of the node maintained by an applied voltage, a peculiar prolonged response may be initiated in the node by superimposing a depolarizing stimulus on the applied voltage. This response consists of a spike followed by a prolonged depolarization which remains for a considerable period of time and decays slowly to the resting membrane level(1,2). Although other explanations have been put forward(1,2), the Ionic Theory of nerve activity actually predicts such a response to be elicitable under these abnormal conditions(3,4).

The Ionic Theory postulates 2 "carrier" systems: one which reacts rapidly with sodium, the other more slowly with potassium during excitation. These systems both depend for activation upon the magnitude of the electrical potential across the cell membrane. At the resting level, the systems are relatively inactive. With reduction of the membrane voltage by depolarization, carrier activity is initiated. The sodium carrier causes the membrane to become selectively permeable to sodium while the potassium carrier actively increases membrane permeability to potassium. Consequently membrane depolarization results in ionic current flow of each specie of ion along its electrochemical gradient. Initiation of sodium carrier activity produces a rapid regenerative depolarization by inward sodium current flow. The initial membrane potential level, –80 millivolts, tends to shift rapidly toward the sodium equilibrium potential, +40 millivolts. This large polarization change inactivates the sodium system, and the outward potassium current flow along its electrochemical gradient drives the membrane potential back toward the normal potassium equilibrium potential, –90 millivolts, and to the original resting level.

What happens in the abnormal situation

when a nerve fiber is immersed in solution containing excess potassium chloride and the membrane potential is held at the resting level by an applied voltage? First of all the potassium equilibrium potential is reduced. For example, the equilibrium potential for 60 millimolar excess potassium solution is almost zero. A voltage applied to maintain the membrane potential near the normal resting value, *e.g.*, –80 millivolts inside, should induce potassium ions to flow *inward* to increase the inside to outside concentration ratio. Such an inward flow would continue until the membrane potential value approximates the equilibrium potential. However, according to the theory, the carrier systems are relatively inactive as long as the membrane potential is held at or near the resting level. Therefore, in spite of the large discrepancy between membrane and equilibrium potentials, the inward potassium current would be small in the above example. A sudden depolarization by an abrupt decrease in the applied voltage should result in activation and a rapid increase in selective permeability to sodium and potassium. Both ions should flow *inward* and depolarize the membrane. The faster reacting sodium system should produce a rapid regenerative spike deflection as in the normal situation. Then the slower reacting potassium carriers should produce a regenerative, long lasting depolarization of the membrane toward the new potassium equilibrium potential level. Consequently, if the membrane voltage of a single fiber immersed in excess potassium solution were clamped at the resting level and then suddenly shifted to a depolarized value, only an inward current comprising two phases, one developed by the rapid transient sodium carrier activity and the other by the slow system associated with potassium, should be observed.

The present investigation was set up to record, if possible, such a membrane current in a single Ranvier node of a nerve immersed in a solution containing normal sodium and high potassium concentration.

Methods. Nerve fibers of frog, *R. Pipiens* and toad, *Bufo Marinus*, were dissected and

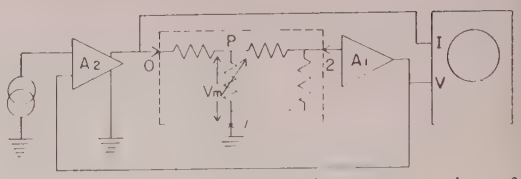

FIG. 1. An electrical schematic representation of the frog single nerve fiber set up for voltage clamp recordings. See text.

mounted in a chamber as described previously(5). Membrane current recordings were obtained by use of a voltage clamp system similar to others(6,7). Figure 1 shows an electrical schematic representation of the single nerve fiber set up for voltage clamping. The numbers 0, 1 and 2 indicate the three electrodes connected with the nodes N_0, N_1 and N_2 respectively. (See Figure 1(5)). The membrane potential of the test node, N_1, is represented by the voltage, V_m, across the variable resistor as shown. The other resistances include the internodal longitudinal resistance and a leakage resistance between electrode 2 and ground. The recording electrode 2, for membrane potential measurements is connected with the input of amplifier, A_1. The output of this amplifier connects directly with the input of the vertical amplifier for one sweep of the recording oscilloscope and with one side of the input of differential amplifier A_2, the clamping amplifier. The other input of amplifier A_2 connects with the stimulator, the output with node N_0 and the vertical amplifier of the other sweep of the recording oscilloscope. Any variation in the voltage V_m, is immediately recorded through the longitudinal fiber resistance by amplifier A_1, the resulting output signal from this amplifier fed back to amplifier A_2 and subsequently into the fiber as a compensating current which re-establishes the original voltage V_m immediately. The compensating current is actually recorded as a voltage developed between electrodes 0 and 1 or, in other words, the voltage output of amplifier A_2 measured between output and ground.

Hypertonic Ringer solutions containing 30-90 millimolar excess potassium chloride were used for most of the experiments. It was found first that solutions containing 2 times

normal concentrations of sodium chloride caused no deleterious effect on electrical activity. The effect of hypertonicity of the excess potassium chloride solutions was, therefore, not considered significant.

The potassium excess solutions were made up by adding potassium chloride to normal Ringer to bring the total amount in solution usually up to 60 millimolar. Since one millimole potassium chloride is equal to 77 mg and normal Ringer contains 2 millimoles potassium chloride, 58 × 77 mg solid potassium were added to one liter Ringer solution.

In some experiments potassium chloride was substituted for sodium chloride in Ringer solution (high potassium isotonic Ringer). To make up these solutions the ingredients sodium chloride, potassium chloride, calcium chloride, sodium bicarbonate, and potassium bicarbonate were made up in separate 117 millimolar isotonic solutions. Then test solutions were made up as follows: normal Ringer: 2 cc KCl, 1 cc $CaCl_2$, 114 cc NaCl, total 117 cc Ringer, buffer to 7.4 with $NaHCO_3$. 60 mM high potassium isotonic Ringer: 60 cc KCl, 1 cc $CaCl_2$, 56 cc NaCl, total 117 cc, buffer with $NaHCO_3$. For 100% isotonic Ringer, sodium chloride completely substituted by potassium chloride, potassium bicarbonate was used for buffering.

The temperature of the preparation mounted in the chamber was maintained at 15-18°C in which range the nerve fiber remained in good condition for many hours.

Results. A typical experiment, results of which are shown in Fig. 1, was as follows: A single fiber was dissected and mounted in the chamber and Ringer solution allowed to flow past the node. The condition of the fiber was examined carefully by noting a normal "threshold" for stimulation and a normal action potential response. Then the feed back system for voltage clamping was switched into the circuit and, without applied voltage, the membrane potential was clamped at the resting level. That is, the clamping amplifier was balanced for zero output with the resting node connected to the input through the potential amplifier(6). The flow system was then switched from Ringer to excess potassium solution and the single node rapidly immersed in this solution. This did not affect the clamped resting membrane potential level but a small inward current was sometimes seen. The membrane potential was then shifted to a depolarized level by a pulse applied to the clamping amplifier input. The current recordings obtained at 3 different voltage clamp levels are shown in Fig. 1, A, B, C. Each picture (A, B, C) in the figure shows 2 traces. The upper trace indicates the current flow, a downward deflection denoting an inward current direction (note reference line). The lower trace indicates the clamped voltage, an upward deflection showing depolarization of the membrane. In Fig. 1, A, with a shift of the voltage clamp in the depolarization direction the current deflection first shows a small artifact and then a slowly developing prolonged inward current. Increased depolarization B, results in an early large spike-like inward current, not seen in A, followed by the slow prolonged inward current. The latency of the slow current has decreased. With still greater depolarization, C, these same 2 inward currents are discernible but merged together. The recording appears as one inward current comprising 2 phases. At this depolarization level the initial spike-like phase of the inward current oscillates. The reason for the apparent merging of 2 separate currents is a decrease in latency of the slower current with increasing depolarization. This decrease in latency is made clear by comparing the onset time of the slow current in each picture. In picture C 2 sweeps have been superimposed, one with and one without depolarization for reference. It is important to note the continuation of the slow inward current after the membrane potential level has returned to the original value.

The ionic currents producing the deflections shown can be directly identified by varying the ionic content, sodium and potassium, of the test solutions. Detailed results of a series of experiments devised for this purpose will be reported later. Briefly it

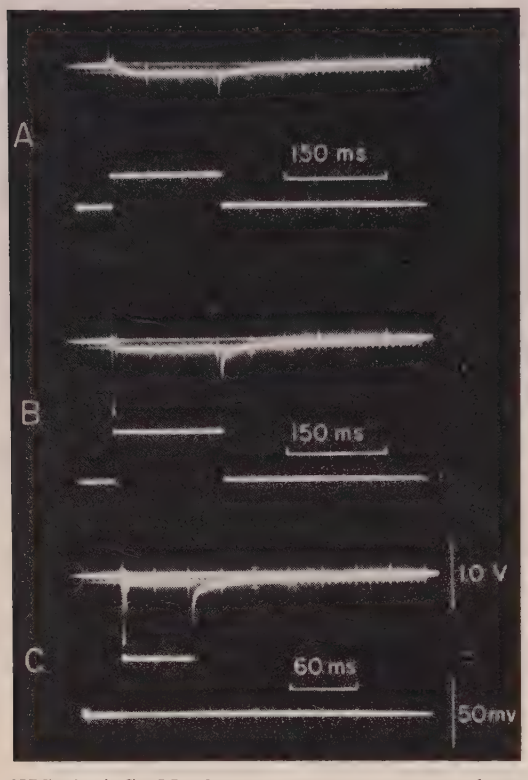

FIG. 2, A-C. Membrane currents, upper tracings, and membrane voltage, lower tracings, recorded from a single Ranvier node in excess potassium solutions under voltage clamp. A. Slowly developing inward current (downward deflection) during abrupt 25 millivolt, 150 millisecond depolarization of membrane. Note prolonged inward current after membrane potential reset to original resting level. Normal base line drawn in white ink for reference. B. Rapid short duration spike-like inward current followed by slow inward current with 40 millivolt, 150 millisecond depolarization of membrane. Normal base line drawn in white ink for reference. C. Oscillatory initial current followed by slow inward current with 50 millivolt, 60 millisecond depolarization. Two sweeps superimposed, one without depolarization for reference. The current measurement is actually voltage output of clamping amplifier and is about 0.25 volts in all the records. Assuming an overall resistance of 50 megohms, the slow currents shown in Fig. 2, A, B, C, are about 5×10^{-9} amps, the transient spike about 2×10^{-8} amps.

can be stated here that in the absence of sodium (100% potassium chloride isotonic Ringer), the early inward spike-like current deflection was never seen. Thus the early rapid transient inward current is to be associated with sodium in accord with other reports(4,6,7). This conclusion is further substantiated by potential recordings which under similar conditions showed only the slow prolonged depolarization of the membrane without the initial spike. The slow inward current could be obtained only with the fiber immersed in high potassium concentration solutions. The magnitude of the current varied directly with potassium concentration. In normal Ringer solution only a small outward current is obtained during continual depolarization(4,6,7). Thus the slow current is to be associated with an inward flow of potassium ions across the node membrane. The 2 currents, sodium and potassium, are apparently independent, though initiation and development of each is related to the membrane potential level as predicted by the theory.

Discussion. Apparently it is possible to record 2 inward currents in the single nerve fiber membrane under a voltage clamp in excess potassium Ringer solution. The independence of these 2 currents or current sources is clearly shown by the fact that one can be initiated without the other. This suggests that there are 2 carrier systems which, when activated, cause the membrane to become selectively permeable to sodium and potassium ions respectively.

The sodium system reacts rapidly whereas the potassium system is relatively slow. The evidence certainly indicates that the prolonged potential response observed in a fiber in excess potassium chloride solution cannot be attributed to a broadening of the action potential *per se*. Two depolarizations, first the sodium spike and then a slow potassium depolarization potential, overlap and comprise the peculiar response observed under such abnormal conditions. This explanation conforms with the Ionic Theory of nerve activity(4).

Summary. The response of the frog single nerve fiber immersed in Ringer solution containing excess potassium has been studied under a voltage clamp. Two clearly discernible inward ionic currents have been observed. These two currents have been associated with sodium and potassium respectively. The evidence presented strongly supports the presently accepted Ionic Theory of nerve activity.

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In vitro Inhibition of Glycogenesis by D-α-Tocopherol.* (26348)

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During a recent study on the glycolytic cycle in Vit. E-deficient rabbits(1,2), it was observed that D-α-tocopherol lowered glycogen levels and lactic acid production in muscle. Zierler *et al.*(3) reported a similar finding for rat diaphragm after *in vivo* administration of α-tocopheryl phosphate. On the basis of data from several pertinent experiments, these authors concluded from indirect evidence that the phosphorylated tocopherol inhibited at the phosphoglucomutase site. It was the purpose of the present investigation to study more directly the action of free tocopherol on glycolysis.

Experimental. Colonies of white, New Zealand rabbits were reared on a Vit. E-deficient diet as previously reported(4). Half the animals (control) received oral supplements of DL-α-tocopheryl acetate and muscle strips were prepared as previously described(1). Oxygen uptake was determined for one hour in presence or absence of D-α-tocopherol with the usual Warburg technics. The tocopherol solution was prepared immediately before use by homogenizing 10 mg of the compound in 0.2 ml of propylene glycol in a 1 ml all glass homogenizer. At termination of the one hour incubation interval, suitable aliquots of tissue were used for estimation of glycogen and lactic acid(1). Isooctane extractions of the medium after incubation were shown to contain 70 to 90% of the added tocopherol as the intact chroman or para quinone by their characteristic ultraviolet absorption.

D-α-tocopherol was also tested on individual purified enzyme systems containing commercially available phosphorylase a, glucose-6-phosphate dehydrogenase and hexokinase. Phosphoglucomutase was also studied and this material was generously supplied by Dr. O. Bodansky.

Phosphorylase a activity was followed by estimating the release of inorganic phosphate while absorption at 340 mμ by reduced triphosphopyridine nucleotide was used to measure the activity of glucose-6-phosphate dehydrogenase(1). The hexokinase reaction was studied by determining the disappearance of glucose according to the method of Nelson(5) as modified by Somogyi(6). The phosphoglucomutase procedure was that of Bodansky(7) in which the disappearance of glucose-l-phosphate (or inorganic phosphate) was followed.

Results. The *in vitro* influence of tocopherol on glycogen levels and lactic acid production in rectus femoris of rabbit is shown in Table I. In the presence of exogenous glucose (0.1 ml of a 10% solution) tocopherol caused a distinct decline (19 to 43%) in glycogen levels in both control and Vit. E-deprived animals. This fall in titer of muscle glycogen was not completely alleviated by increasing the rate of glucose-6-phosphate synthesis from glucose. Addition of 1 mg hexokinase and 1 mg adenosine triphosphate

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TABLE I. *In Vitro* Effect of D-α-Tocopherol on Glycogen and Lactic Acid Levels of Skeletal Muscle.

Condition of animal	No. of animals	Additions	Glycogen, mg/100 mg dry wt* Added tocopherol None	1 mg	% change	Lactic acid, mg/100 mg dry wt* Added tocopherol None	1 mg	% change
Control	6	None	1.44 ±.11	.86 ±.02	−40	.65 ±.08	1.78 ±.16	+174
"	"	1 mg hexokinase + 1 mg ATP	1.95 ±.11	1.32 ±.15	−32	.81 ±.11	2.11 ±.13	+160
Deficient	5	None	.98 ±.10	.56 ±.04	−43	.61 ±.08	1.49 ±.09	+144
"	"	1 mg hexokinase + 1 mg ATP	1.19 ±.10	.97 ±.18	−19	.67 ±.04	1.67 ±.15	+150

* Mean ± S.D.

stimulated glycogen deposition on the average 29% for control and 64% for deficient animals. However those tissues exposed to tocopherol still gave lower glycogen values.

An estimate of lactic acid formation showed an increase in muscle lactate ranging from 144 to 174% (Table I) for all groups. Introduction of hexokinase had a similar slight effect on lactic acid production in both groups of animals. Lactate content was increased by 22 to 24% for control and 10 to 12% for deficient muscle. However the difference between tocopherol-treated and untreated groups remained unchanged.

That a specific concentration of tocopherol was necessary to decrease muscle glycogen levels can be seen in Fig. 1. Glycogen depletion was discernible at tocopherol levels of 0.6 mg for 250 mg wet weight of skeletal muscle. The picture was the same for control and Vit. E-deficient rabbits.

The same figure shows the *in vitro* effect of tocopherol on oxygen uptake by skeletal muscle from control animals. There is a steady and significant increase. Addition of tocopherol to Vit. E-deficient muscle results in a decrease in respiration of the muscle(1). It is to be recalled that oxygen consumption in muscle from Vit. E-deprived animals may be elevated from a slight to a marked degree (reviewed in reference 4).

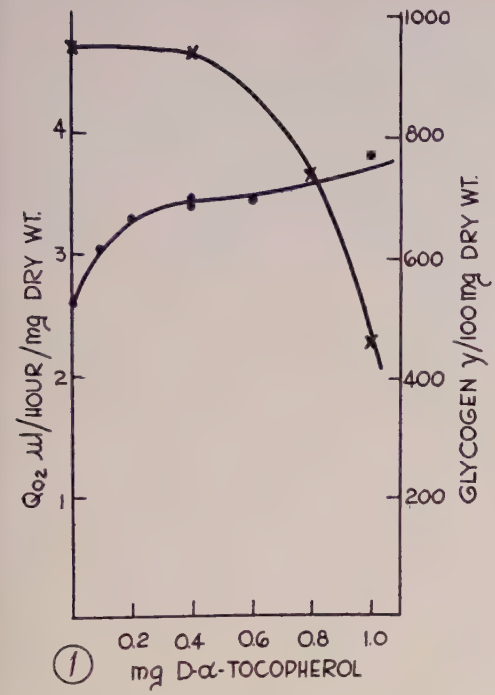
Extension of this investigation to purified enzyme systems demonstrated that tocopherol (0.5 to 1.0 mg) had no influence on the phosphorylase a system, on conversion of glucose-6-phosphate to 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase or on the hexokinase system. However in the instance of phosphoglucomutase, conversion of glucose-1-phosphate to glucose-6-phosphate proceeded to approximately 90% in the control incubation and to only 70% in presence of tocopherol (Table II).

The extent of tocopherol interference with the function of phosphoglucomutase became more apparent when the enzyme level was varied. Results of this experiment are depicted in Fig 2. The control system without tocopherol required 3 μg of enzyme per ml of reaction mixture to reach maximum transfer of the phosphate group from position 1 to 6. This level of enzyme was completely inhibited by 1 mg of tocopherol. Doubling the enzyme titer released the inhibition by approximately 70%. No inhibition was ob-

TABLE II. Effect of D-α-Tocopherol on Purified Phosphoglucomutase.*

Enzyme	Tocopherol added, μg	Density	% change
None	0	.38	
"	1000	"	
Phosphoglucomutase	0	.04	
"	500	.08	−12
"	1000	.14	−30

* Glucose-1-phosphate disappearance was estimated in a solution containing 0.1 ml of 0.03 M $MgSO_4$, 1 ml of 0.1 M tris (hydroxymethyl) aminomethane, 0.5 ml of phosphoglucomutase (30 μg/ml in 0.1 M histidine), 0.5 ml 0.025 M glucose-1-phosphate and 0.4 ml of water. 1 ml of reaction mixture was added to cold 5 ml of 5% trichloroacetic acid and after 5 min. a 1 ml aliquot was diluted with 3 ml of water, 1 ml of 5 N H_2SO_4 and heated in a boiling water bath for 5 min. To the cooled solution was added 3.6 ml of water, 1 ml of 2.5% ammonium molybdate and 0.4 ml of the Fiske-SubbaRow reagent.



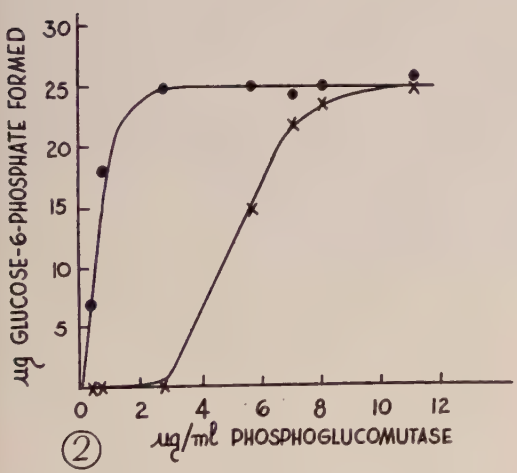


FIG. 1. Effect of varying concentrations of D-α-tocopherol on glycogen (×——×) and oxygen uptake (●——●).

FIG. 2. Formation of glucose-6-phosphate with varying concentrations of phosphoglucomutase in absence (●——●) and presence (×——×) of one mg D-α-tocopherol.

tained at phosphoglucomutase concentrations above 9 μg/ml of the reaction mixture.

Discussion. Zierler *et al.*(3) in an early work had found that tocopheryl phosphate decreased oxygen consumption of rat diaphragm by 25%. This effect on respiration was abolished by addition of glucose or certain members of the Krebs' cycle. Therefore these investigators interpreted the results to mean that tocopheryl phosphate exerted its influence between glycogen and glucose-6-phosphate. Additional evidence from the ability of tocopheryl phosphate-treated diaphragm to dissimilate glycogen and convert glucose-6-phosphate toward lactate led these workers to indicate phosphoglucomutase as the site of tocopheryl phosphate inhibition.

In the present study it was more directly demonstrated that tocopherol could partially inhibit phosphoglucomutase and therefore glycogenesis or glycogenolysis. In the presence of sufficient quantities of glucose-6-phosphate from exogenous glucose or due to added hexokinase, the influence of tocopherol on phosphoglucomutase could be obscured. Some of the excess glucose-6-phosphate can be converted to glycogen while glycolysis leads to an increased production of lactate.

Although it has been reported that activity of phosphoglucomutase declines in human muscular dystrophy(8) and in mice with inherited dystrophy(9), tocopherol levels in blood and tissues were within normal limits. However Carpenter, *et al.*(10), using a Vit. E-deficient diet which permitted rabbits to live 100 days, found no change in level of phosphoglucomutase. The importance of tocopherol in glycogenesis through its influence on phosphoglucomutase remains to be determined.

Summary. The *in vitro* influence of D-α-tocopherol on glycogen levels, lactic acid formation and oxygen uptake has been studied in rectus femoris muscle from control and Vit. E-deficient rabbits. It was shown that less than one mg quantities of tocopherol decrease glycogen stores, increase lactic acid formation in both groups of animals and increase oxygen consumption in control muscle. In purified enzyme systems it was found that tocopherol inhibited phosphoglucomutase and not phosphorylase, hexokinase or glucose-6-phosphate dehydrogenase.

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Effect of Hemorrhagic Shock on Viability of Invading Bacteria.* (26349)

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There is now substantial evidence that bacterial endotoxins play a deciding role in development of irreversibility to transfusion in various types of traumatic shock(1,2). Much of this evidence incriminates the intraintestinal bacteria or their endotoxins, which are absorbed from the intestinal lumen into the circulating blood in the normal as well as the shocked animal(3). In the normal animal the endotoxin is taken up and detoxified by the R.E. system, so that there is no detectable amount in the circulation. The R. E. system of the shocked animal can take up very little, and of that it cannot detoxify more than a small fraction(4). The endotoxin is, therefore, free in the circulation to inflict damage on the peripheral vessels, and so to prevent recovery from shock.

It is of considerable importance to know to what extent the gram-negative bacteria in the tissues, whether they are invaders from the gut or contaminants of wounds, might contribute to the amount of circulating endotoxin. The data to be presented show (1), that bacteria regularly traverse the intestinal barrier; and (2) that even though they multiply when the antibacterial defenses are down, their number in the first filtering depots (liver and mesenteric lymph nodes) is too small to contribute significantly to the endotoxemia.

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Methods. With the use of aseptic technic and sterile equipment, healthy adult white rabbits, with a normal intestinal flora, were given heparin and then bled from a femoral artery, cannulated under local anesthesia, into an elevated reservoir, so as to produce a continuous arterial pressure of 50 mm Hg(5). Two groups (B & C) were maintained at this pressure for 1½ hours, and a third group (D) for 6 hours, after which all the shed blood was returned. Four or 8 hours after this replacement transfusion, a sterile laparotomy was performed under ether anesthesia. The abdomen was prepared for laparotomy by burning the alcohol-soaked skin. Separate sets of instruments were used to incise the skin, to open the peritoneum, to biopsy the ileal lymph nodes, and the liver.

The biopsies were placed in sealed vials of brain heart infusion broth for transfer to the laboratory. Before culturing the samples were removed from the containers, placed in Petri dishes, and exposed to ultraviolet light for 15 minutes to destroy any surface contaminants. They were then ground in a tissue grinder with either brain heart infusion broth or trypticase soy broth. The resultant suspension was inoculated on heart infusion blood agar plates containing 10% defibrinated horse blood, and into brain heart infusion broth and Brewer thioglycollate broth. One set of cultures was incubated aerobically and another anaerobically in Brewer jars under 95% N_2 and 5% CO_2. In most in-

TABLE I. Incidence of Positive Cultures of Mesenteric Nodes and Livers of Normal and Shocked Animals.

Group	Type of exp.	No. of animals	No. of livers with		No. of mesenteric nodes with		No. of animals with	
			Gram-neg. rods*	Gram-pos. rods and cocci	Gram-neg. rods*	Gram-pos. rods and cocci	Gram-neg. rods*	Gram-pos. rods and cocci
Rabbits								
A.	Control	19	0	2	0	7	0	9
B.	1½ hr shock 4 hr after transfusion	7	1	4	3	7	3	7
C.	1½ hr shock 8 hr after transfusion	5	5	3	3	3	5	5
D.	6 hr shock 8 hr after transfusion	3	3	1	2	2	3	2
Dogs								
	Control	10	4	5	7	8	8	8
	2 hr shock 8 hr after transfusion	5	2	4	4	3	4	4

* Gram-negative cocci were found only once in the entire study and so were omitted from the table.

stances, the broth from the transporting container was incubated aerobically. All cultures were incubated at 37°C for 7 days. All tubes and plates showing growth were studied further. Bacteria were examined for morphology and gram reaction. Broth cultures and representative colonies from plates usually were subcultured aerobically and anaerobically on blood agar.

Cultures were considered positive only when organisms were isolated from nodes or liver which differed from any bacteria grown in the broth of the transporting container. Growth in the container was considered to be attributable to surface contamination of the biopsy.

The mesenteric nodes and livers of a control group (A) of rabbits were studied by the same methods as those of the rabbits in Groups B, C and D. Several of the rabbits were subjected beforehand to arterial cannulation, and to 6 hours of restraint in the supine position, but without producing shock, to determine to what extent the manipulation attending induction and maintenance of the shock, rather than the shock itself, may have contributed to bacterial invasion of the nodes and liver.

Similar studies were made on dogs, as indicated in Table I.

Results. (Table I). *A. Rabbits.* Control Group A—The mesenteric lymph nodes and livers of 19 normal rabbits yielded not a single positive culture for gram-negative organisms. Clostridia and gram-positive enterococci were present occasionally. Of the 12 rabbits transfused after 1½ hours of hemorrhagic shock, 7 (Group B) were studied 4 hours afterwards. Three showed gram-negative rods. There was also an increase in incidence of gram-positive organisms. The remaining 5 (Group C) were studied 8 hours afterwards. All 5 showed gram-negative rods, as well as a moderate increase in incidence of gram-positive bacteria. Group D consisted of 3 rabbits in hemorrhagic shock for 6 hours. All 3 showed gram-negative rods in the liver 8 hours after transfusion. Thus, whereas gram-negative bacteria were not detected in any normal rabbit's tissues, 11 of 15 (73%) of shocked rabbit's tissues were positive for these bacteria. Their incidence was higher the longer the interval between transfusion and cultures. The percent of shocked animals with gram-positive organisms was considerably greater than that of normal rabbits with these organisms.

B. Dogs. Contrary to the findings in

rabbits, the incidence of gram-negative bacteria in 10 normal dogs was very high, as was also the case for gram-positive bacteria (80%). The same incidence was found in 2 hour shocked dogs 8 hours after transfusion. Organisms did not grow on the primary plates in the cultures from control dogs but did grow on subculture from broth. Bacteria grew on the primary plates from the shocked dogs, suggesting the possibility that the numbers of organisms in the tissues of these animals was greater than in normal animals.

Discussion. The presence of gram-negative bacteria in the liver and other tissues of the dog was investigated by Jacob *et al.* (6), who reported that these bacteria were rarely found in normal dogs examined immediately after death, whereas gram-positive rods and cocci were found frequently. But when the tissues of dogs subjected to 6 hours of shock were examined immediately after death, there was a sharp increase in incidence of gram-negative as well as gram-positive bacteria. Jacob *et al.* suggested that the bacteria found immediately after death in the tissues were not simply "agonal invaders," but were bacteria that normally and regularly invade from the gut, and that if they could be found only immediatley post-mortem it was because the anti-bacterial potential was adequate until death. The higher incidence of such bacteria in the shocked as compared to the normal dog was taken to signify *in vivo* decline in defense potential. This decline had been demonstrated by showing that the shocked animal cannot destroy a number of intravenously injected gram-negative or gram-positive bacteria, which the normal animal can kill with ease, and that this disability persists beyond 24 hours in the animal recovering from shock(7).

When endotoxemia was shown to be a primary factor in development of irreversibility to transfusion, attention was again focussed on the gram-negative bacteria from which the endotoxin is derived. Even though the evidence pointed to those in the intestine as the major, if not the sole, source of the circulating endotoxin(3), the possibility that those which had invaded and were alive in the tissues might be contributing to the endotoxemia needed reinvestigation. From the results of the present study with the use of more meticulous technics than were employed in the earlier study, the previous report(8) that the gram-negative bacteria cannot be cultured from the living tissues of the dog must be revised.

Their presence in most normal and shocked dogs signifies that they, like the gram-positive bacteria, are continuous invaders from the gut. The fact that they were recovered from the tissues of the normal dog only on subculture, but were recovered from the tissues of the shocked dog without subculturing, suggests the possibility of increased numbers of bacteria in shocked dogs attributable to the decrease in defense potential, which has been repeatedly demonstrated in other ways.

The same general principle applies in the rabbit: Bacteria which cannot be grown out of the normal rabbit's tissues can be found in shocked rabbits with increasing incidence the longer the interval after shock during which they are allowed to multiply. This increasing incidence cannot be attributed to greater bacterial invasiveness during shock, for this would require the finding of a peak incidence during shock, rather than during the recovery period.

In spite of the evidence that gram-negative as well as gram-positive bacteria continuously invade from the gut *via* the lymphatics and portal vein, and that these bacteria persist or multiply during and for some time after shock because of the decline in anti-bacterial potential, the number of colonies growing are so few that it is not possible to account for any significant fraction of the endotoxin in the circulating blood of shocked animals from this source. This conclusion is based on the following data: One gram (wet wt.) of *E. coli,* containing approximately 2×10^{10} to 1×10^{11} bacteria, grown under near optimal cultural conditions, yields 10-20 mg of partially purified endotoxin by the TCA extraction technic of Boivin and Mesrobeanu. Thus a single bacterium can make up to 1 to

2×10^{-7} μg of endotoxin. A rabbit weighing 2 kg and fed 2-3 g (wet wt.) of an identifiable (tagged) strain of *E. coli* will yield some 200 μg of labelled endotoxin from its liver and blood alone(3). If all this were derived from bacteria in the tissues there would have to be approximately 200,000 gram-negative bacteria per gram of tissue in all organs. These bacteria have been found only in the mesenteric nodes and liver, and in only an insignificant fraction of this number.

Studies of the bacteria which may contaminate the groin wound made for cannulating the femoral vessels to induce and maintain shock by the reservoir technic have been made repeatedly. When the experiment is performed with clean though not sterile technic (*i.e.* with use of autoclaved instruments, shaved skin, thoroughly clean glassware, tubing, etc.) bacteria in the wound at the end of experiment are the usual skin contaminants, *i.e.*, staphylococci, *B. subtilis*, diphtheroids and an occasional Clostridium. Gram-negative bacteria are not found. The lack of relevance of these bacteria to the course of events in the rabbit exposed to severe and prolonged hemorrhagic shock is shown by the fact that endotoxemia, irreversible shock and death occur in spite of the use of aseptic technic, or of locally applied antibiotics (Polymyxin and Bacitracin), which prevent bacterial proliferation, as demonstrated by wound cultures at the time of death(9).†

Summary and conclusions. Gram-positive bacteria can be grown from the mesenteric nodes and liver of the normal rabbit and dog. Gram-negative bacteria can be grown from these tissues of the normal dog, but not of the normal rabbit. Gram-negative bacteria are found in the nodes and liver of the rabbit some hours after exposure of the rabbit to 2 hours of hemorrhagic shock. The incidence is higher 8 hours afterward than it is 4 hours afterward. These data confirm the view that bacteria are continuously invading from the intestine. Exposure for 2 hours to hemorrhagic shock so weakens the antibacterial defense that gram-negative as well as gram-positive bacteria can be grown out more readily than in the normal animal. However, the number of bacteria that can be recovered is judged too small to be a significant contribution to the endotoxemia of advanced hemorrhagic shock. Reasons are given for also excluding the bacteria normally found in the groin wound, made for cannulating the femoral vessels, from any significant role in the endotoxemia.

† Schweinburg and Fine(10) state that when such animals are transfused after 2 hours in shock they are hypersensitive to endotoxin because of damage to the R. E. system by the shock. Greisman confirms the occurrence of hypersensitivity to endotoxin, but he attributes it to the combined effects of the hemorrhagic shock and infection in the groin wound (11). This conclusion is based on his view that the groin wound is unavoidably infected with Clostridia, even when aseptic technic is used, because the dissection done to cannulate the vessels activates the Clostridia in the adjacent tissues. Since the Clostridia he finds in these wounds are Perfringens, a strain not found in rabbit tissues, it is clear that his experimental model consists of overt induced sepsis as well as hemorrhagic shock. The mortality in his experiments may be due to the complicating infection rather than the endotoxin. Greisman's experimental model is not comparable to ours, which, as stated, does not result in overt infection. His failure to find hypersensitivity in the rabbits put into hemorrhagic shock by cardiac puncture may be accounted for by the considerably lesser degree of shock induced by this method than by the reservoir technic, for the hypotension part of the time is as high as 60 mmHg, and the intervals allowed between successive bleedings to produce the shock allows a considerably greater mobilization of fluid than is likely with the reservoir technic.

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Utilization of Serum α_1-Globulin and Glycoprotein by *Diplococcus pneumoniae*.* (26350)

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Changes of the serum protein fractions and of the bound carbohydrate (glycoproteins) of these fractions are known to occur in many pathological conditions. However, little is known about the metabolic alterations which lead to these changes, about the normal synthesis and destruction of glycoproteins in the animal body. Information is also lacking concerning destruction of the blood glycoproteins by micro organisms. A preliminary survey in this laboratory involving a number of micro organisms revealed a surprising resistance of serum glycoproteins to bacterial destruction. Data reported by Friedemann and Sutliff(1), however, indicate that the pneumococcus may utilize the protein bound carbohydrate of sera from individuals with certain pathological conditions.

In recent experiments, a strain of *Diplococcus pneumoniae* was utilized which had been isolated from a patient with pulmonary pneumonia at University Hospital, Oklahoma City, and maintained in frozen stock.† Five ml of sterile serum was transferred to a 50 ml sterile Erlenmeyer flask and inoculated with one loopful of 6-hour, rapidly growing culture of the organism. The inoculated serum sample was incubated at 37°C for 20 hours. A control serum sample, similar in all respects except uninoculated, was incubated for the same time in the same way. After incubation, the bacterial cells were removed by centrifugation. Determinations of protein were made on the supernatant by the biuret method; glycoprotein hexose was determined by the method of Shetlar *et al.*(2). Two samples of bovine sera, 2 rat samples, and 8 human samples (4 normal, 2 cancer, 2 rheumatoid arthritis) were investigated. The inoculated bovine samples decreased from 151 to 130 mg per 100 ml in glycoprotein content, and from 7.13% to 6.46% in protein content. Similar changes were noted in rat sera. Results of the human studies are summarized in Table I. In every instance, samples incubated with the organism decreased in both protein and glycoprotein content. When the normal human samples were considered, the difference between serum glycoprotein contents of inoculated and control groups was statistically significant at the 1% level; the decrease of the protein content was also significant at the 1% level.

These data indicate the preferential destruction of carbohydrate-rich protein by the micro organism. This is confirmed by paper electrophoretic studies of protein and glycoprotein of the sera by methods previously described(3). Results of a typical study of serum from a cancer patient are shown in Fig. 1. Results of α_1-glycoprotein determined by paper electrophoresis are given in Table I. It appears from the electrophoretic pattern and from the results in Table I, that the micro organism preferentially utilizes α_1-glycoprotein. Similar results were found in paper electrophoretic investigations of all human samples and of rat and bovine sera. The decrease of α_1-globulin averaged 85% (ranging from 50-100%);

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† The authors are greatly indebted to Dr. Florene Kelly and Mr. Billy West, Dept of Microbiology, for the culture and for advice on bacteriological technics.

TABLE I. Protein and Glycoprotein Changes of Human Sera after Incubation with *Diplococcus pneumoniae.*

Condition	Total protein, g/100 ml		Total glycoprotein, mg hexose/100 ml		α_1-Globulin glycoprotein, mg hexose/100 ml	
	Organism*	Control	Organism	Control	Organism	Control
Normal (4)	6.64 ±.07†	6.93 ±.08	99 ± 3.1†	117 ± 3.5	0	19
Carcinomatosis	4.75	5.04	134	174	12	62
Breast cancer	7.12	7.62	152	176	12	38
Rheumatoid arthritis	6.10	6.66	129	180	0	45
Idem	5.87	6.19	158	182	9	32

* Serum incubated with organism for 20 hr at 37.5°C. Control incubated in same way at same time.

† Significantly lower than control value at 1% level. Values following ± signs are stand. errors of mean.

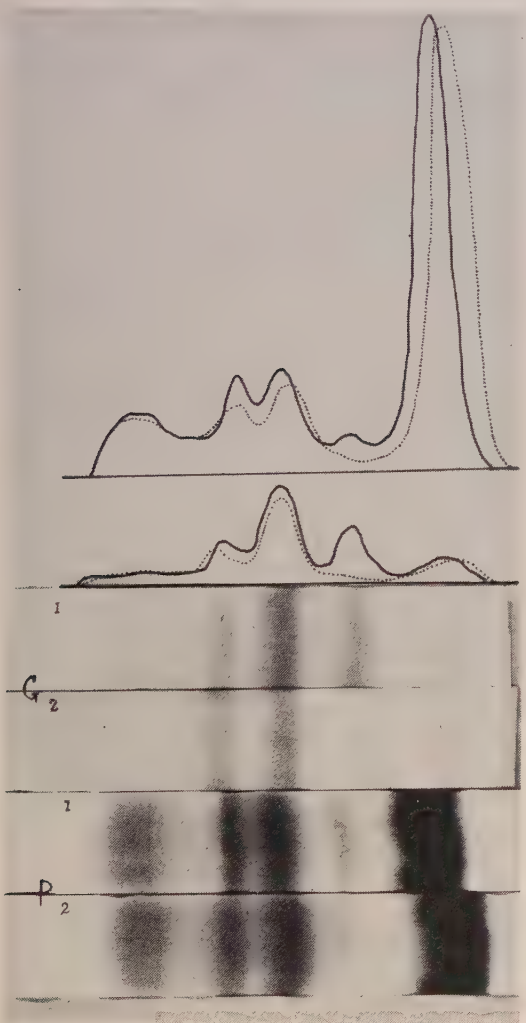

FIG. 1. Alterations of electrophoretic patterns of human serum after incubation with *Diplococcus pneumoniae.* Lower 2 strips (marked "P") are stained for protein with bromphenol blue. No. 1 is of control (incubated without organism); No. 2 is strip of the sample after incubation with microorganism. Note decrease of α_1-globulin fraction (second band from right). Upper strips (marked "G") are corresponding strips stained by the periodic-acid-Schiff procedure for glycoprotein. Again the α_1 fraction is nearly absent in sample incubated with the organism (No. 2). Above both strips are densitometer tracings of these patterns. Protein tracings (from "P") are at top; immediately below are glycoprotein tracings. In both cases, control tracing shown by solid line; those from samples incubated with the organism shown as dotted lines. Note decrease of α_1-globulin peaks in each case.

when considered in absolute terms (milligrams per 100 ml of serum), the decrease of α_1-globulin hexose was approximately equal to the loss of total glycoprotein hexose. No other fraction was appreciably affected.

Further studies are required to determine whether the α_1-globulins (or other globulins) from the serum of patients with certain diseases are used more readily by pneumococcus than those from normal sera. It seems likely, however, that the increased lactic acid production by the pneumococcus from sera of patients with pathological conditions as previously described(1) may be due to increased α_1-globulin in these sera. Elevations of the α_1-globulins and the glycoproteins of this fraction are known to occur in these diseases.

These studies are of interest in connection with the recent report of Kent and Gey(4) which indicated a preferential utilization of α_2-globulins by rat tumor cells. Of wider importance is the possibility that enzyme systems may be isolated from the pneumococcus which will be useful in degradation studies of the glycoproteins.

Summary. When serum samples were in-

cubated with a rapidly growing strain of *Diplococcus pneumoniae,* striking decreases of serum glycoprotein and consistent decreases of protein resulted. Paper electrophoretic studies indicated that these changes were largely restricted to the α_1-globulin fraction. It would appear that this strain of *Diplococcus pneumoniae* preferentially uses glycoproteins found in the α_1-globulin fraction of mammalian serum.

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Endemic Toxoplasmosis in Isolated Swine and Cattle Herds and Its Relationship to a Human Population.* (26351)

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Serological findings have shown that toxoplasmosis occurs as an attenuated infection in man and many warm blooded animals(1, 2,3). Rarely does overt disease occur with *T. gondii* when one considers the high incidence of infection. The means of natural transmission remain unknown(4). The present study presents additional information for natural transmission from animal to animal and from animal to man in an isolated environment.

In a recent study of 703 patients at the Georgia Training School for Mental Defectives,† Gracewood, Ga., serologic procedures had pinpointed a high incidence of attenuated infection in 2 of 12 cottages. In these 2 cottages 51% of 86 patients were positive to the toxoplasmin skin test as compared to 15.4% of 617 patients in other cottages. The only related identifiable factor which differentiated these 2 cottages was that they housed the patients who assisted in the farm work and in the care of the farm animals. Each of the 12 cottages had a relatively stable population which slept in that cottage and ate together to form a "family group." A central kitchen prepared the food for all cottages; milk was purchased from an outside dairy. There was a central water supply.

Investigation of infection in the 2 large animal herds maintained at Gracewood revealed the high incidence shown in Table I.

The farming program at Gracewood was under the direction of a graduate agriculture engineer and all animals were under the medical supervision of a licensed veterinarian. No epidemic or unusual disease of acute or subacute character had occurred among the animals for the past 9 years. The herds investigated served as a source of beef and pork for the Training School; all meat passed U. S. Government standards. The swine herd had been kept for over 12 years in a pasture adjacent to the slaughter house. The beef cattle were pastured with the swine until 1957 when they were removed to new pastures 5 miles distant; however, cattle were still occasionally pastured with the swine at intervals to control the forage crops. The pasture was plowed twice yearly to minimize parasite infestation. All garbage collected from the Training School was heated to 212°F for a minimum of 30 minutes before being fed to swine. Warfarin was placed out routinely for rodent control.

Isolation of T. gondii. To substantiate serological incidence of infection in the swine herd, isolation attempts were made on the brain tissue of 14 randomly selected hogs that were slaughtered for table consumption.

* This investigation was supported by grant from Nat. Inst. Health.

† Appreciation is expressed for the cooperation of the Gracewood Training School administrative staff.

TABLE I. Methylene Blue Dye Test.

Animal	Herd size	Animals tested	Dilution 1:16		Dilution 1:64	
			No. pos.	% pos.	No. pos.	% pos.
Swine	350	48	33	68.8	25	52.1
Cattle	440	24	20	83.3	12	50.0

The viable organism was demonstrated in the brains of 5 of these animals. The brain tissue was collected within 3 hours after slaughter and processed within an additional 2-24 hours. Thirty grams of brain tissue were homogenized in a Waring Blendor with the gradual addition of 100 cc isotonic saline. The homogenate was filtered through 4 layers of gauze, then centrifuged in 100 cc amounts at 1200 rpm for 30 minutes. The supernate was removed and the sediment was thinned with 10 cc of saline before 0.75 cc was inoculated intraperitoneally into each of 6 Webster mice weighing 20-24 g. Cortisone was given subcutaneously in amounts of 2.5 mg on day of inoculation and on each of the 2 following days. Two strains were identified and maintained by mouse passage and 3 other identifications were made from Wright stains of peritoneal exudates. Early mouse passage required a 6-day interval in this host before successful transfer. After 2 months of laboratory passage, transfers could be made following a 3-4 day interval.

Discussion. The high incidence of naturally occurring attenuated infection with *T. gondii* in 2 domestic herds on the Gracewood farm identified a circumscribed area of endemic infection. It was considered highly improbable that the average incidence of toxoplasmosis in herds of domestic animals would be as high as 60-80%. Feldman and Miller(5) studied 5 cattle herds and found that incidence of toxoplasmosis varied from 0 to 49%. The 83% incidence in the herbivorous herd on Gracewood Farm indicated that transmission from animal to animal had occurred very successfully by means other than consumption of infected meat. Furthermore, the high incidence (51%) of toxoplasmosis in the patients in only 2 out of the 12 cottages in which all patients were supplied from the same meat source, indicated that consumption of infected meat was not the primary mode of transmission. The known absence of overt disease and the known high incidence of infection as shown by serological testing indicated that the strain of *T. gondii* isolated at Gracewood was of low virulence under conditions of natural transmission.

Summary. A localized area in Georgia has been identified that showed a high incidence of attenuated infection with *T. gondii* in a herd of cattle and of swine. This locale provided a means of investigating the epidemiology of this infection under natural conditions. The high incidence (51%) of toxoplasmosis in the patients of 2 cottages was related to association with the infected animals but was not related to consumption of infected meat.

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Effect of Several Inorganic Salts on Infectivity of Mengo Virus.* (26352)

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There have been few systematic investigations of the effect of salts on inactivation of small animal viruses(1). Columbia SK, a strain of encephalomyocarditis (EMC) virus, was noted to be unstable when diluted in physiological saline(2). EMC virus was also inactivated in dilute chloride salts but was relatively stable in distilled water(3). Ribonucleic acid derived from Mengo infected tissues was biologically and physically more stable in hypertonic sodium chloride than in isotonic sodium chloride(4). This paper presents a study of inactivation of the Mengo strain of EMC virus in sodium chloride and certain other salts.

Materials and methods. Chemicals were all analytical reagent grade. Solutions were made with distilled water passed through a mixed bed ion exchange resin. Concentrations are expressed as molar (M) or millimolar (mM).

Mengo strain of EMC virus(5) was obtained through the courtesy of Dr. A. E. Moore. A single pool of 43rd mouse passage was prepared as a 20% W/V mouse brain homogenate in distilled water containing a final concentration of penicillin 80 units/ml and streptomycin 0.16 mg/ml. After centrifugation at 5000 g for 1 hour, the supernate was divided into 1 ml aliquots, sealed in glass ampules, frozen and stored at –70°C.

Serial 10-fold dilutions of virus were made using chilled diluents and glassware. Each dilution tube was subdivided into 2 or more 0.9 ml aliquots, thus making 2 or more replicate sets of each dilution series. One such set was immediately tested for infectivity in mice. The others were incubated in a water bath, without shaking, for times and temperatures indicated in the respective experiments, then tested for infectivity.

Titrations in 16-18 g Swiss-Webster mice were by intraperitoneal inoculation, 0.05 ml inoculum per mouse, 5 mice per dilution, using 5 successive dilutions chosen to include the LD_{50} as judged by previous experiments. Deaths occurred as early as day 2 and seldom later than day 5. LD_{50} dilutions were calculated from deaths through the 7th day(6).

Results. Replicate dilutions of Mengo-infected mouse brain in 150 mM sodium chloride were tested for infectivity before and after varying periods of incubation at 37°C. There was a progressive loss of infectivity during the first hour, with a consistent inactivation of approximately 10,000 LD_{50} units per unit volume by the end of 2 hours (Table I). The persistence of infective virus at 10^{-3} dilutions even after 2 hours of incubation probably represents the protective effect of 0.1% mouse brain extracts(3).

Parallel titrations incubated at different temperatures indicated that loss of infectivity varied directly with temperature of incubation (Table II). Relatively little inactivation occurred at 0°C in contrast to inactivation at 37°C. This result is in agreement with the study of Boesche and Drees(3) on EMC virus although a different method was used, and confirms the original observation of Jungeblut and Sanders(2).

Replicate dilutions of Mengo virus in distilled water and in various concentrations of

TABLE I. Results of Incubating Replicate Serial Dilutions of Mengo for Various Times in 150 mM NaCl at 37°C, and Injecting Intraper. into Mice. Fractional figures represent No. of mice dead/No. of mice inj.

Virus dilution	Time of incubation 0′	15′	30′	60′	90′	120′
10^{-2}			5/5	5/5	5/5	5/5
10^{-3}		5/5	5/5	4/5	5/5	4/5
10^{-4}		5/5	5/5	1/5	1/5	0/5
10^{-5}	5/5	3/5	1/5	0/5	0/5	0/5
10^{-6}	5/5	0/5	0/5	0/5	0/5	0/5
10^{-7}	4/5	0/5				
10^{-8}	1/5					
10^{-9}	0/5					
LD_{50}	$10^{-7.5}$	$10^{-5.2}$	$10^{-4.6}$	$10^{-3.5}$	$10^{-3.6}$	$10^{-3.4}$

* Supported in part by research grants from Nat. Cancer Inst., Bethesda, Md.

TABLE II. Effect of Temperature on Infectivity of Mengo Incubated in 150 mM NaCl for 2 Hours.

Temp., °C	Virus LD_{50} $-\log_{10}$ 0′	120′
37	7.5	3.4
32	7.8	4.5
27	7.7	5.3
22	7.6	5.6
0	7.8	6.6

sodium chloride were tested for infectivity before and after incubation for 2 hours at 37°C. The virus was stable in markedly hypertonic sodium chloride and distilled water. Maximal inactivation occurred between 15 mM and 150 mM (Table III).

There was little or no inactivation of Mengo virus diluted and incubated in several other sodium salts (Table IV). The presence of $Na+$ in concentrations of 150 meq/l is not, in itself, a sufficient condition for inactivation. Monobasic sodium phosphate (pH 4.3) and dibasic sodium phosphate (pH 9.0) represent the minimum and maximum pH of diluents used in these studies suggesting that inactivation observed in the present studies is not primarily an effect of pH.

When replicate dilutions of Mengo virus in four chloride salts were tested for infectivity before and after incubation at 37°C for 2 hours, there was marked inactivation in both monovalent and divalent salts (Table V). Attempts to modify the degree of inactivation by varying the ratio of monovalent to divalent cation(7,8) have so far been unsuccessful.

TABLE III. Effect of Different Concentrations of NaCl on Infectivity of Mengo Incubated at 37°C for 2 Hours.

Diluent	Virus LD_{50} $-\log_{10}$ 0′	120′
DW	8.3	7.5
NaCl .15 mM	7.7	7.5
" 1.5	8.7	≥8.2
" 15	7.6	3.8
" 50	7.8	3.4
" 75	7.6	3.5
" 100	7.8	2.8
" 150	7.6	3.6
" 300	7.6	5.4
" 500	7.4	≥7.0
" 1000	7.6	7.3
" 1500	8.0	7.2

Inactivation in sodium halide salts occurred most extensively in chloride and bromide, somewhat less in iodide, and very little in fluoride, the pseudo halogen sodium thiocyanate, or distilled water (Table VI). At-

TABLE IV. Effect of Sodium Salts on Infectivity of Mengo Incubated at 37°C for 2 Hours.

Diluent	Virus LD_{50} $-\log_{10}$ 0′	120′
NaCl 150 mM	7.7	3.5
$NaClO_4$ "	8.4	7.5
$NaNO_3$ "	8.7	7.8
NaH_2PO_4 "	7.5	6.5
Na_2HPO_4 "	7.8	7.1
Na_2SO_4 "	7.8	7.2
Na formate "	8.2	6.8
Na acetate "	7.8	6.5
DW	8.3	7.5

TABLE V. Effect of Chloride Salts on Infectivity of Mengo Incubated at 37°C for 2 Hours.

Diluent	Virus LD_{50} $-\log_{10}$ 0′	120′
NaCl 150 mM	7.5	3.4
KCl 150	8.0	3.5
$MgCl_2$ 75	7.8	3.5
$CaCl_2$ 75	7.1	3.5
DW	8.5	7.8

tempts to differentiate between bromide and chloride by varying time and temperature of incubation were unsuccessful within the sensitivity of this method. Iodide was consistently less inactivating than either chloride or bromide and, with the lack of inactivation in sodium fluoride, may represent a dependence of inactivation on anionic size or hydration.

Discussion. The use of purified virus for

TABLE VI. Effect of Sodium Halide Salts on Infectivity of Mengo Incubated at 37°C for 2 Hours.

Diluent	Virus LD_{50} $-\log_{10}$ 0′	120′
NaF 150 mM	8.3	7.4
NaCl "	7.8	3.5
NaBr "	7.6	3.6
NaI "	7.7	4.5
NaSCN "	7.0	6.5
DW	8.2	7.6

inactivation studies has obvious advantages. However, crude virus preparations are convenient for use in a preliminary survey such as this, the information gained may be usefully applied to further purification procedures, and some of the inherent disadvantages of crude virus preparation may be lessened by the choice of methodology.

Diluting a concentrated crude virus preparation to a convenient lesser concentration in a diluent of known composition(3,7) decreases the concentration of non-viral material—thus possibly decreasing the influence of such chemically ill-defined material on the type of inactivation under study(9). A further advantage of diluted virus preparations is the increased sensitivity in detecting absolute changes in infectivity by assaying an all or none response to further serial dilutions. If rate of inactivation is not directly dependent upon virus concentration, as might occur in certain types of enzymatically limited reactions, such problems of assay sensitivity may be significant. Incubating serial dilutions of virus made in sodium desoxycholate and then testing for infectivity is an even more sensitive method used by Theiler(10), upon which the method of the present study is based. It is usually quite apparent (Table I) that an LD_{50} lies between 2 of the dilutions tested. However, application of the classic method of Reed and Muench(7) to such data the calculation of what would be an LD_{50} after incubation under the specified conditions, is admittedly an approximation.

The intraperitoneal route for titrations in these studies was chosen because of the toxicity of certain of the diluents when administered to mice intracerebrally, or when used in tissue culture.

It is of interest that inactivation is maximal at approximately the chloride concentrations of the extracellular fluid. It is conceivable that inactivation by chloride at body temperature is a mechanism of host defense. Whether inactivation is a direct effect of chloride on the virus or is mediated through some tissue component is unknown. The stability of this virus in both distilled water and hypertonic salt may in part determine the circumstances under which it is transmitted and persists in nature.

Summary. Mengo infected mouse brain preparations were stable, as judged by mouse lethality, when diluted in 1 molar sodium chloride or distilled water and incubated at 37°C. Mengo virus was inactivated when incubated in 150 mM sodium chloride. Inactivation was directly related to time and temperature of incubation and was demonstrable at concentrations of sodium chloride between 15 and 300 millimolar. Inactivation appeared to be related to the presence of Cl–, or similar anions, rather than Na+.

The author gratefully acknowledges the advice and encouragement of Dr. Chester M. Southam, helpful discussions with Dr. M. Earl Balis, and invaluable assistance of Miss Katherine Aliminosa.

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In vitro Metabolism of Specifically Labeled Glucose by Adipose Tissue of Mice Bearing Adrenocorticotropic Tumors.* (26353)

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Adrenocorticotropic tumors, induced in mice surviving exposure to ionizing radiations from an atomic detonation, have been implanted in successive passages and are now autonomous in that they can be grown in non-adrenalectomized hosts(1). Normal mice grafted with such tumors are submitted to high levels of endogenous glucocorticoids and study of the metabolism of their isolated tissues might give valuable information on the mode of action of the natural adrenocortical secretion. Adipose tissue is a major site of hormonal regulation(2,3) and it seemed therefore worthwhile to compare the metabolism of this tissue in tumor-bearing mice and in untreated controls.

Material and methods. Male mice of the LAF_1 strain, fed Ralston-Purina laboratory chow *ad libitum,* had been injected 4 weeks previously in the thigh muscles with a saline suspension of tumor tissue. Terramycin HCl was added to their drinking water in order to control the infections which are frequently observed in mice with adrenocorticotropic tumors. The animals were approximately 14 weeks old at time of sacrifice. Untreated mice of the same strain and age served as controls. The epididymal fat pads were excised with minimal handling and the metabolic activity of one fat pad, incubated without insulin, was compared to the activity of its paired control, incubated in presence of insulin (0.1 U/ml of recrystallized insulin containing less than 0.1 μg glucagon per mg, obtained through the courtesy of Dr. W. R. Kirtley, Lilly Research Labs, Indianapolis, Ind.). Incubations were carried out at 37°C, in 3 ml of Krebs-bicarbonate buffer, for 3 hours. Glucose concentration was 20 mM/liter and glucose radioactivity was one microcurie per flask (chromatographically pure glucose-1-C^{14} or glucose-6-C^{14} obtained from Nuclear Chicago Corp.). The methods used in determining the amount of glucose carbon oxidized to CO_2 or incorporated into long chain fatty acids, alpha carbon of glyceride-glycerol, and glycogen, have been described (4,5). All values have been expressed in terms of micromoles of glucose carbon metabolized per mg of tissue nitrogen per 3 hours, as well as in terms of percentage of total amount of recovered glucose carbon.

Results. In the grafted mice, the adrenocorticotropic tumors measured approximately one cm in diameter. Adrenal weight was roughly double that of the controls. The effect on body weight was variable (Table I). However, mean weight of the 2 epididymal

TABLE I. Fatty Acid and Nitrogen Content of Epididymal Adipose Tissue from Mice with Adrenocorticotropic Tumors.

		Mean values for epididymal adipose tissue (both pads)				
		Weight		Fatty acids,	Nitrogen	
Type and No. of mice	Body wt range (g)	mg	% of body wt	% wet wt tissue	mg	% wet wt tissue
Controls (6)	21-31	340	1.2	78	.59	.17
Tumor-bearing (11)	20-35	530	2.0	79	.63	.12

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‡ Recipient of a Research Fellowship from Dazian Foundation for Med. Research, New York.

TABLE II. *In Vitro* Metabolism of Glucose-1-C^{14} and Glucose-6-C^{14} by Epididymal Adipose Tissue from Mice with and without Adrenocorticotropic Tumors. (Krebs bicarbonate buffer. Glucose concentration 20 mM. Insulin concentration, when present, 0.1 U/ml. Values expressed as micromoles (μM) of glucose carbon metabolized per mg of tissue nitrogen per 3 hr, or as percentage (%) of total recovery. Mean values ± S.E.M.)

Type and No. of mice	Insulin added *in vitro*	Carbon dioxide				Fatty acid				Glyceride-glycerol alpha carbons				Glycogen				Total			
		μM		%		μM		%		μM		%		μM		%		μM		%	
		C-1	C-6	C-1	C-6	C-1	C-6	C-1	C-6	C-1	C-6	C-1	C-6	C-1	C-6	C-1	C-6	C-1	C-6	C-1	C-6
Control (6)	—	1.53 ±.21	.58 ±.10	57	29	.46 ±.14	.70 ±.29	17	35	.67 ±.15	.66 ±.16	25	33	.03 ±.01	.05 ±.02	1	3	2.69	1.99	100	100
	+	9.90 ±2.31	.55 ±.05	66	5	3.57 ±.61	8.15 ±1.08	22	76	.92 ±.18	1.33 ±.23	6	12	.75 ±.09	.68 ±.18	5	6	15.1	10.7	100	100
Tumor-bearing (11)	—	.95 ±.14	.71 ±.09	58	44	.16 ±.05	.29 ±.10	10	18	.51 ±.14	.61 ±.15	31	38	.01 ±.01	.01 ±.01	1	1	1.63	1.62	100	100
	+	4.90 ±.89	1.49 ±.43	61	27	1.28 ±.26	1.73 ±.39	16	32	1.67 ±.33	2.16 ±.78	21	39	.17 ±.04	.15 ±.03	2	3	8.02	5.53	100	100

fat bodies was found to be somewhat greater in the tumor-bearing mice and represented 2.0% of total body weight, as compared with 1.2% in normal mice. Nitrogen content was lower in the epididymal adipose tissue of tumor-bearing animals, while fatty acid content was the same in both groups. The *in vitro* metabolism of carbon atom-1 and carbon atom-6 of glucose by isolated epididymal adipose tissue is shown in Table II. Total recovery of glucose carbon-1 and glucose carbon-6 was lower in the adipose tissue of tumor-bearing mice, being respectively 66% and 81% of that observed in control animals. Furthermore, the metabolism of these 2 glucose carbons was modified. Incorporation of glucose carbon-1 and glucose carbon-6 into glycogen was depressed, and their incorporation into fatty acids reduced to less than half. Recovery of both glucose carbons in the alpha carbon of glyceride-glycerol was normal. Oxidation of carbon-1 was depressed to 62% of the norm, while oxidation of carbon-6 was comparable to that observed with control animals. When these values are expressed on a percentage of recovery basis, the relative impairment in lipogenesis remains striking and a relatively high oxidation of glucose carbon-6 becomes apparent.

The effect of insulin added to the medium on metabolism of glucose was also different in the tissue from tumor-bearing and from control mice. In the adipose tissue of mice with adrenocorticotropic tumors, total recoveries of both glucose carbons were only 50% of the norm, and lipogenesis and glycogen synthesis were promoted to a lesser extent by the presence of insulin in the medium. Oxidation of glucose carbon-6, and glycerol synthesis from both carbons, however, were stimulated more markedly in the tissue from the tumor-bearing animals.

Discussion. The metabolic picture prevailing here is the result of a complex endocrine situation. In the tumor-bearing animals, adipose tissue is chronically submitted to high levels of endogenous ACTH, endogenous glucocorticoids (mostly corticosterone(6)), and perhaps also insulin, as suggested by marked hypertrophy of the Islets

of Langerhans(7). Total recovery of glucose carbon-1 and glucose carbon-6 metabolized by the adipose tissue of tumor-bearing animals was lower than normal, suggesting that overall glucose metabolism was less. Relatively greater suppression of oxidation of glucose carbon-1 than carbon-6, with relatively greater suppression of lipogenesis from glucose carbon-6, suggests relatively greater depression of the phosphogluconate-oxidative pathway in adipose tissue of tumor-bearing mice. On the other hand, normal capacity of this tissue for oxidation of glucose carbon-6 suggests relative integrity of the tricarboxylic acid cycle. The results showing an impaired lipogenesis are in line with those obtained in rats treated with cortisone. In these animals cortisone pretreatment has been shown to result in decreased lipogenesis in subsequently isolated adipose tissue (8,9) and also to depress the respiratory quotient of this tissue(10,11). A reduction in responsiveness of glycogenesis and lipogenesis to insulin was apparent when insulin was used in concentrations known to be maximally effective on mouse adipose tissue(5). The presence of altered insulin sensitivity is also suggested by the observation that, in spite of the indirect evidence of hyperinsulinism in the tumor-bearing mice(7), the metabolism of their isolated epididymal adipose tissue differed grossly from what one might expect as a result of chronic insulin overdosage.

It is known that adrenocorticotropic tumor-bearing mice contain 3 times as much extrahepatic fat as their controls, even when body weight is in the normal range(12). If the metabolic data obtained *in vitro* (Table II) may be considered as representative of the situation prevailing *in vivo,* it would seem that this increased accumulation of fat is not likely to be the result of accelerated lipogenesis. Other mechanisms must be involved. Accelerated hepatic lipogenesis with peripheral accumulation of the newly synthesized fat has been suggested by the experiments of Zomzely and Mayer(12). Delayed mobilization of fat from fat stores is also conceivable.

Summary. The *in vitro* metabolism of specifically labeled glucose by epididymal adipose tissue of mice bearing adrenocorticotropic tumors has been studied. Sluggish lipogenesis from glucose was found to be coupled with evidence suggesting decreased activity of the phosphogluconate oxidative pathway. Glyceride-glycerol synthesis was normal, and oxidation of glucose carbon in the tricarboxylic acid cycle appeared to proceed at a normal rate. Insulin added *in vitro* was less effective in promoting lipogenesis and glycogen synthesis than with tissue from control animals.

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Sensitization by Thallium to Dihydrotachysterol Overdosage.* (26354)

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Chronic intoxication with thallium salts produces loss of hair occasionally accompanied by convulsions, skeletal deformities, and clouding of the lens reminiscent of tetanic cataracts. This syndrome has been compared to hypoparathyroidism and was thought to result from some primary damage to the endocrine system(1). However, the voluminous literature on thallium intoxication is rather contradictory, because of the great variability in results obtained by different investigators(2,3).

Recently, we found that highly selective calcification in the cortico-medullary junction line of the kidney is consistently obtained in rats given a single dose of thallium acetate and of dihydrotachysterol (DHT), even though, in the amount administered, neither substance in itself causes nephrocalcinosis. The thallium acetate also accentuates other manifestations of DHT-intoxication, so that a synergism between this vitamin-D derivative and thallium can readily be demonstrated.

Methods. Forty female Sprague-Dawley rats, with an average initial body weight of 95 g (range: 90-102 g), were subdivided into 4 equal groups and treated as indicated in Table I. Thallium acetate, 2 mg in 0.2 ml of water and DHT ("Calcamin," Wander), 500 μg in 0.5 ml of corn oil, were administered subcutaneously and by gavage, respectively, once on the first day. For control purposes, one group of rats was restrained with adhesive tape, in the prone position, during the 24 hours immediately following DHT-administration, to verify that the thallium salt did not act merely by virtue of its stressor effect.

Earlier observations had shown that the trauma of epilation precipitates cutaneous calcinosis in the DHT-sensitized rat(4). To determine whether this action of DHT is likewise aggravated by concurrent treatment with thallium, the hair was removed by plucking over the calvarium in all 4 groups on the second day.

The experiment was terminated on the 8th day and degree of calcification in skin, kidney, aorta, and heart was appraised by loupe-inspection in terms of an arbitrary scale: 0 (no lesion), 1 (just detectable lesion), 2 (moderate lesion), and 3 (maximal lesion). The data were subsequently checked by v. Kóssa's silver nitrate technic on histologic

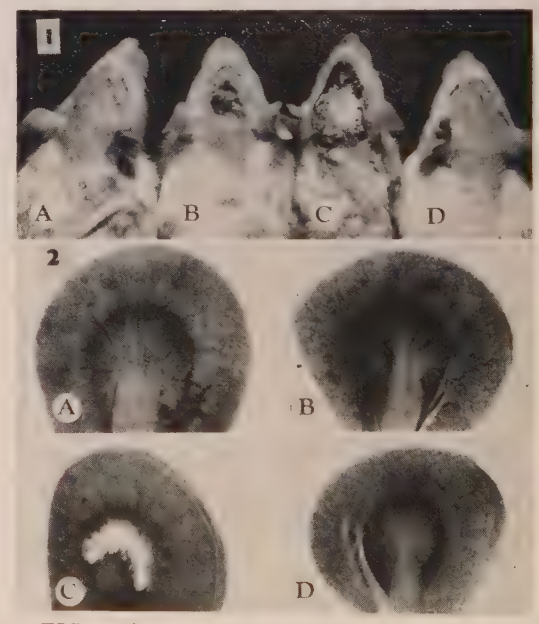

FIG. 1. Appearance of epilated skin area in rats treated with thallium acetate (A), DHT (B), thallium acetate + DHT (C), and restraint + DHT (D). Following treatment with DHT alone, the cutaneous calcinosis is mild, while concurrent treatment with thallium acetate results in formation of a solid, hard plaque of scleroderma-like skin calcinosis. In the remaining 2 animals, no cutaneous lesions are detectable.

FIG. 2. Cross sections through kidneys of rats treated with thallium acetate (A), DHT (B), thallium acetate + DHT (C), and restraint + DHT (D). Only combined treatment with thallium acetate and DHT produces massive selective calcium deposition at the corticomedullary junction line.

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TABLE I. Sensitization by Thallium to Dihydrotachysterol Overdosage.

Treatment	Mean final body wt (g)	Calcification (scale 0-3)			
		Cutaneous	Renal	Aortal	Cardiac
Thallium-Ac	122 ± 1.47	0	0	0	0
DHT	104 ± 3.75	1.8 ± .42	0	.3 ± .15	.6 ± .33
Thallium-Ac + DHT	79 ± 3.12	3.0 ± 0	2.8 ± .15	1.1 ± .20	1.3 ± .41
Restraint + DHT	95 ± 2.10	0	0	0	0

sections of specimens fixed in alcohol-formol. The means of these readings, as well as the mean final body weights, are listed in Table I (with standard errors).

Results. Cutaneous lesions were absent in rats treated with thallium alone and moderate in those receiving DHT alone, while combined treatment with both these agents resulted in maximal cutaneous calcinosis in all animals (Fig. 1). No renal lesions were detectable in rats treated with thallium acetate or DHT, alone; but rats given both these compounds exhibited a clearly demarcated zone of intense calcification in the corticomedullary junction zone (Fig. 2).

Thallium acetate also failed to cause calcification in aorta or heart, however, a slight degree of calcium deposition was detectable in these organs, following treatment with DHT alone. The intensity of the aortal calcification was significantly ($P<0.01$) aggravated by concurrent treatment with thallium acetate; but in the heart, this aggravation was not statistically significant ($P = 0.2$).

The sensitization to DHT afforded by thallium acetate could not be ascribed to the stressor effect of the latter, because our earlier findings showed that pretreatment with stress produced nonspecific cross-resistance against DHT-intoxication(5). This was confirmed under the conditions of the present experimental series by the fact that, far from aggravating the syndrome of DHT-intoxication, 24 hours of restraint actually prevented it.

Summary. A single dose of thallium acetate, which in itself produces no detectable organ lesions in the rat, causes severe nephrocalcinosis strictly limited to the corticomedullary junction line if an otherwise non-nephrotoxic dose of dihydrotachysterol (DHT) is administered simultaneously. The DHT-induced calcification in aorta and in traumatized skin regions is greatly aggravated by concurrent treatment with thallium acetate. This sensitizing effect of thallium acetate cannot be ascribed to its stressor action, since under comparable circumstances, exposure to stress (restraint) actually prevents the manifestations of DHT-intoxication.

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Preliminary Report on a Hemagglutination Test for Entamoebae.* (26355)

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Previous serologic tests for amebiasis have made use of antigens prepared from amoebae grown symbiotically in cultures with mixed or single bacteria from the intestinal tract of man or animals. Such antigens have shown

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inconsistent results(1,3,4,5,6,7,8,10,11,13,15). It therefore seemed desirable to determine the specificity of antigens prepared from entamoebae grown in association with symbionts that do not occur in the intestinal tract. *Trypanosoma cruzi* reported by Phillips(12) to support growth of *E. histolytica* was first selected for such study.

Methods. 1. Preparation of antisera in rabbits. Two amoebae, *E. invadens* and *E. histolytica,* (DKB strain) were selected for preliminary study. The former was chosen because it is morphologically similar to *E. histolytica,* produces pathologic changes in reptiles, and may be grown in axenic culture. It was thought that *E. invadens* might possibly produce an antigen suitable for use in human amebiasis tests. As *E. invadens* did not grow profusely in either Miller's(9) or Stoll's(14) medium in our hands, both of the amoebae were adapted to monobacterial cultures of *Aerobacter aerogenes.* Amoebae of each species were washed as free as possible from the bacteria, suspended in saline, counted and injected into rabbits subcutaneously and intraperitoneally by appropriate schedules for production of homologous antisera. Serum samples were collected from the rabbits before immunization and at intervals until a substantial titer of antibodies was attained.

2. Preparation of antigen for serologic tests. Antigens for serologic tests were produced by growing each species, *i.e., E. invadens,* and *E. histolytica* (DKB strain) with *T. cruzi* in Phillips' medium previously obtained through the courtesy of Dr. Wm. Balamuth. Growths of cultures were harvested and centrifuged at 1000 rpm for 10 minutes in an International #2 centrifuge, and the sediments washed twice in 85% NaCl. Two distinct layers of the sediment were obtained. The upper fluffy white layer consisting mostly of trypanosomes was removed and the gray mucoid sediment that remained, contained 11,500,000 trophozoites of amoebae per milliliter when examined in a Neubauer counting chamber. No cysts were seen. The amoebae were suspended in 10 ml of chilled, distilled water, and ruptured by vigorous passage through a 20 gauge needle. This preparation was allowed to stand in the refrigerator over night, the extract then being restored to isotonicity by adding 10 ml of 1.7% NaCl, and clarified by high speed centrifugation in the cold. The resulting water-clear solution provided a stock antigen at a dilution of 1:20. By using amoebic antigen from cultures grown with *Trypanosoma cruzi* no cross reactions were expected with the *Aerobacter aerogenes* or its medium that had previously been injected into rabbits for production of antisera.

3. Hemagglutination test. A modification of Boyden's(2) original tanned cell hemagglutination procedure, developed by one of the authors, (W.P.L.), for toxoplasmosis work was employed. This involved (a) substitution of human O cells for sheep cells, and (b) tanning the cells at concentrations of tannic acid ranging from 1:80,000 to 1:120,000 at a temperature of 2°C. The antigen was attached to the tanned cells at a pH of 6.4 and a temperature of 37°C.

Four controls were used in the test: (i) serum collected from the rabbits before injection with antigen, (ii) antiserum from rabbits injected with antigen prepared from *A. aerogenes* only, (iii) tanned human O cells with no antigen and (iv) human O cells with *T. cruzi* antigen.

The antigen prepared from *E. invadens* titered to an optimum dilution of 1:40 and the antigen from *E. histolytica* to 1:280.

Results and discussion. 1. The reciprocal reactions of these preliminary hemagglutination tests are shown in Table I.

2. At this point during the investigation a serum from a man with amebic liver abscess was obtained and tested both with the antigen of *E. histolytica,* DKB, and the antigen of *E. invadens.* With the antigen of *E. histolytica* an antibody titer of 1:512,000 was

TABLE I. Homologous and Heterologous Reactions of *E. invadens* and *E. histolytica,* DKB, with Antisera Produced in Rabbits.

Antigens	Antisera	
	E. invadens	*E. histolytica*
E. invadens	2048	32
E. histolytica	128	2048

TABLE II. Hemagglutination Test Studies in Amebiasis.

Sera from persons with	No. sera tested	No. positive	% pos.
1. No suspected amebiasis	30	1*	2.0
2. Three or more stools negative for *E. histolytica*	18	0	
3. Diarrhea or dysentery. *E. hist.* trophs. in stools	37	36	
4. Chronic G.E. symptoms. *E. hist.* in stools	13	13	98.0
5. Amebic liver abscess	12	12	
6. No current clinical amebiasis *E. hist.* in stools Probably "carriers"	34	23	68.0†

* Had traveled in Mexico.

† Most "carriers" were from tropical areas.

obtained while with the antigen prepared from *E. invadens* an antibody titer of only 1:256 was obtained. It was therefore evident that the common antigenic relationship existing between *E. histolytica,* DKB, and *E. invadens* as judged by the current hemagglutination test is slight and that further studies to determine its applicability to human amebiasis should be made with antigen prepared from *E. histolytica.*

3. Preliminary application of hemagglutination test to human sera. Sera tested to date have been procured from Los Angeles, and from abroad through the courtesy of Dr. Lei Kian Joe of Djakarta, Indonesia; Dr. Molina Pasquel, Inst. de Salubridad Y Enfermedades Tropicales, Mexico City; Dr. T. C. Backhouse, School of Public Health and Tropical Med., Sydney, Australia; and Dr. Elsdon-Dew, The Amoebiasis Research Unit, Durban, South Africa. Groupings of the sera tested, number of sera from each group and number and percentage positive are recorded in Table II.

The following comments may be made. a. Only one, or 2% of 48 control sera from persons without clinical amebiasis or whose stools are negative for *E. histolytica* was positive by the hemagglutination test. This person had previously traveled in the tropics.

b. Sixty-one or 98% of the sera from 62 persons with clinical amebiasis showing either a current history of dysentery or diarrhea, chronic intestinal amebiasis or amebic liver abscess, yielded a positive hemagglutination test. The one negative serum was drawn 6 days after onset with dysentery which was the shortest period after onset of any case reported in our series. (See Note(†), Table III).

c. Sera from persons who came to the hospital with complaints other than amebiasis but whose stools were positive for *E. histolytica* showed 68% to have a positive hemagglutination test. Most of these individuals were from Indonesia. Judging from the report of Magath and Meleney(8) "carriers" in the United States show a low percentage of posi-

TABLE III. Correlation of Hemagglutination and Complement Fixation Tests in Amebiasis.

*Reciprocals	Complement fixation titers*									Totals	A.C.‡
512,000								1	1	2	1
128,000						1	3		2	6	1
32,000						1	1	2	1	5	0
8,000	2	2	1	3	1	1	2			12	4
2,048	2	1	3	1	2	2	2			13	5
512	3	3	2	1	1					10	4
128	7			1						8	3
32	4	1								5	0
8	2									2	0
Neg.	46			1			1†			48	8
	Neg.	8	16	32	64	128	256	512	1024	111	26

† Taken 6 days after onset of disease.

‡ Many of the sera tested had been forwarded by mail from overseas.

tive complement fixation tests for amebiasis and it is quite possible that when sera from additional "carriers" living in temperate zones are studied, a lower percentage of positive hemagglutination tests will also be evident. This would indicate that *E. histolytica* is at times present in the human bowel without sufficient tissue invasion to stimulate antibody production.

d. Of special interest is a 38-year-old woman, continuously resident either in Michigan or California, who exhibited a mass in the colon thought to be malignant. A partial colectomy was performed. The removed mass was determined by the pathologist to be an ameboma. The serum from this patient taken a few days after surgery showed a hemagglutination titer of 1:32,000.

e. Three individuals with amebic liver abscess showed a 4-fold drop in hemagglutination test titer in 8 months after therapy and one person with chronic amebiasis showed a drop in titer from 1:128 to negative 2 months after treatment.

4. Complement fixation test. The same antigen for *E. histolytica* used in the hemagglutination test described above was employed in the complement fixation test performed in accordance with the standard procedure recommended by the Virus Laboratory, Calif. State Dept. of Public Health. It has been used at a dilution of 1:320, based on net weight of the amoebae, and was not anticomplementary when tested in a dilution of 1:80. Table III shows data obtained from 136 sera tested, 111 of which were satisfactory by both tests. As many of these sera had been shipped from overseas without refrigeration and had been stored for long periods of time, an unusually high number were anticomplementary and could not be included in this comparison. From the 111 that were satisfactory, it will be observed that a. 46 of 48 sera testing negative by the hemagglutination test were also negative by the complement fixation test. b. Of 7 sera positive with titers of 1:8 and 1:32 by the hemagglutination test only 1 gave a positive complement fixation test. c. Of 42 sera positive by the hemagglutination test at titers between 1:128 and 1:8000, 14 gave negative complement fixation tests. The remaining 28 or 67% were positive at titers ranging from 1:8 to 1:256. d. All hemagglutination tests positive to titers of 1:32,000 or above were positive by the complement fixation test. Such complement fixation test titers ranged from 1:128 to 1:1024.

It will be observed that the antigen prepared from *E. histolytica* DKB works equally well in the hemagglutination test and the complement fixation test, moreover there is no evidence of cross reaction with the *T. cruzi* controls, all of which were negative. In areas where Chagas disease occurs it is possible that positive control sera from persons infected with *T. cruzi* may be encountered.

The antigen showed no evidence of being anticomplementary when used in the complement fixation test, thus avoiding a problem often encountered by previous workers. As it is probable that this same antigen may be used successfully in other serologic tests for amebiasis, experiments to determine its chemical composition are planned.

Summary. Antigens prepared from *E. invadens* and *E. histolytica,* DKB strain grown in monomicrobial culture with *T. cruzi* in Phillips' medium have demonstrated high sensitivity and been used successfully in reciprocal hemagglutination tests with specific antisera prepared in rabbits. The antigen prepared from *E. histolytica* has also been used in both the hemagglutination test and the complement fixation test employing sera from individuals with and without amebiasis. In the hemagglutination test 98% of the former were positive while only 2% of the latter sera were positive. Sera from "carriers" most of whom lived in the tropics, gave 68% positive correlation.

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Suppression of Virus-Induced Corneal Toxicity in Rabbits by Pretreatment with Nitrogen Mustard.* (26356)

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When a large amount of Newcastle disease virus (NDV) is inoculated into the anterior chamber of the rabbit eye, there results a toxic reaction characterized by corneal opacity and characteristic microscopic changes of corneal endothelium(1,2). No evidence of increase in infectious virus is associated with the above reactions. Since an extensive infiltration of leucocytes, particularly polymorphonuclear leucocytes (PMN), into ocular tissues as well as aqueous humor, is a constant feature, it was considered possible that the infiltrating cells might play an essential role in the pathogenesis of the corneal reaction. Because of the known suppressive effect of nitrogen mustard on PMN *in vivo,* this agent was employed in an effort to study the above possibility.

Materials and methods. Virus: The L-Kan 1948 strain of NDV, a heat resistant strain(3) obtained from Dr. H. Rubin, was used in all experiments. It was cultivated in 10-day-old embryonated eggs by inoculating 10^4 plaque forming units (PFU) of stock virus into the allantoic cavity. The allantoic fluid harvested at 48 hours was pooled and centrifuged at 3000 rpm for 5 minutes to eliminate cell debris. It was dispensed into glass tubes in a volume of 1 ml per tube and tightly sealed and stored at –35°C until used. The titer of the virus in this allantoic fluid was 2×10^9 PFU with a hemagglutinin titer of 1280 units per ml. NDV infectivity was determined by using the plaque count technic on cells derived from chick embryo (4).

Rabbit. Normal healthy male albino rabbits weighing between 4 and 4.5 lb were used.

Nitrogen mustard (HN_2). Mustargen hydrochloride (Methyl-bis [beta-chloroethyl] -amine HCl) (Merck, Sharp and Dohme, Philadelphia, Pa.) was used. It was dissolved in sterile double-distilled water just before injection into rabbits.

Intraocular injection of virus. Rabbits were anesthetized with intravenous sodium Nembutal. Two minutes prior to intraocular injection of virus, a drop of Pontocaine HCl (Winthrop Labs, N. Y.) was topically applied to the cornea. The technic for injection of the inoculum into the anterior chamber of the eye has been described(2).

Preparation of corneal endothelium for microscopic examination. Corneal endothelium was stained with a solution of silver nitrate and an entire sheet of stained endothelium was separated from the cornea and mounted on a slide according to methods described previously(2).

Methods of scoring corneal lesions. 1) *Endothelial rosettes.* Microscopic lesions on the silver stained endothelium of the cornea, referred to as endothelial rosettes, were counted by examining the entire endothelium

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of the cornea mounted on a slide, with a high dry objective at a final magnification of 430. Lesions were recorded as total number of rosettes counted per cornea.

2) *Endothelial fusions.* Other microscopic lesions on the silver stained endothelium, called fusions, were scored as follows after examining the entire endothelium mounted on a slide under the microscope with a final magnification of 430: − = no fusion on entire sheet of endothelium, ± = scattered fusions of 2 cells, + = frequent fusions of 3 to 5 cells, ++ = fusions so extensive that only a few cells with cell boundaries are seen in a field, +++ = cell boundaries lost almost completely.

3) *Corneal opacity.* The gross change of the cornea, opacity, was usually scored 24 hours after virus inoculation into the anterior chamber, and at other appropriate times. Degree of corneal opacity was scored as follows: − = no opacity, ± = doubtful opacity, + = definite but weak opacity, outline of iris can be seen easily through cornea, +-+ = intermediate degree of opacity between + and +++, ++-+ = strong opacity, outline of iris cannot be seen through cornea.

Counting leucocytes in aqueous humor. A drop of aqueous humor was placed on a slide, air dried and stained with Wright's solution. The entire drop of aqueous humor was examined under the microscope with a final magnification of 430, and total number of leucocytes present was obtained. Corneal endothelial cells were also seen in aqueous humor as single cells or as groups of cells. These corneal endothelial cells were not included in total leucocyte count of the aqueous humor.

Experimental results. Suppression of leucocytes in blood by intravenous injections of HN_2. This preliminary experiment demonstrated the effects of HN_2 on circulating leucocytes. Six rabbits were injected intravenously twice at an interval of 2 days with HN_2, 0.75 mg per lb of body weight. Total leucocyte and differential counts were carried out at various intervals, on blood withdrawn from the marginal ear vein. Care was taken to avoid unnecessary irritation to the ears. One ear was used solely for HN_2 injection and the other for sampling blood. To prevent bacterial infection during the prolonged period of leucocyte depletion, each rabbit was injected intramuscularly with 10,000 units of procaine penicillin and 10,000 μg of streptomycin given daily for 7 days starting from day of first HN_2 injection. The results of the experiment are summarized in Fig. 1.

The number of circulating leucocytes reached the lowest level, ranging from 400 to 750 per cmm, 2 days after the second injection of HN_2. This severe leucopenia lasted for 2 to 4 days. Then a sharp increase in number of leucocytes followed and there was a return to an almost normal leucocytic level on the 14th day after the first injection of HN_2. Mononuclear cells were shown to be the first cells affected by the HN_2 injection; a drastic decrease to as low as 6% of total leucocytes was noted 24 hours after first HN_2 injection. The relative proportion

TABLE I. Effect of Intravenous Injection of HN_2 on Virus-Induced Corneal Toxicity.

HN_2 injections			Corneal reaction (opaque corneas/corneas used)	
No. of injections	Days before inj. of virus	Exp. No.	HN_2-treated rabbits	Control rabbits*
2	4 and 2	1	0/4	4/4
		2	1/12	5/8
		3	2/6	6/6
		4	0/2	6/6
		5	0/8	6/8
		6	1/4	2/3
		7	1/6	6/6
		Total	5/42	35/41
	2 and 0	1	0/6	6/6
		2	4/6	5/6
		3	4/6	6/6
		Total	8/18	17/18
1	4	1	6/6	5/6
		2	4/4	6/6
		3	5/6	4/6
		Total	15/16	15/18
	2	1	7/8	7/8
		2	3/6	5/6
		3	4/6	6/6
		Total	14/20	18/20
	0	1	6/6	6/6
		2	4/6	5/6
		3	4/6	6/6
		Total	14/18	17/18

* HN_2-untreated.

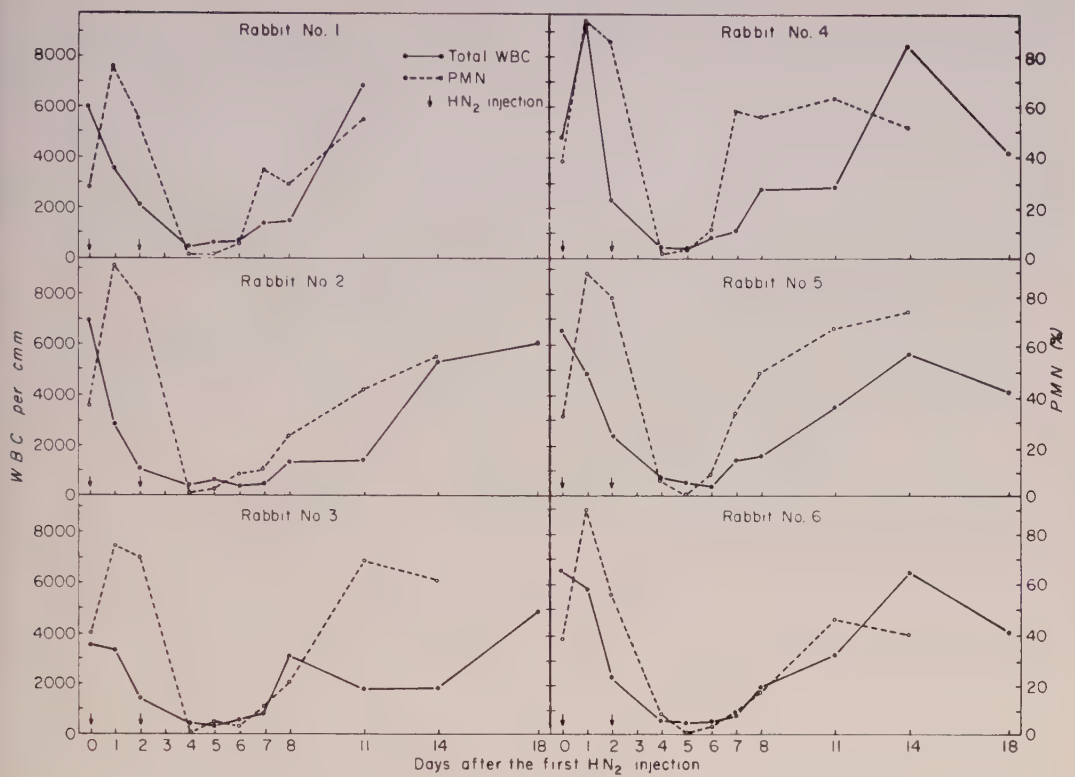

FIG. 1. Suppression of leucocytes in rabbits by 2 intrav. injections of HN_2.

of PMN reached the lowest level, ranging from less than 1% to 8%, 2 days after the second injection of HN_2 and remained at that level for 2 to 3 days. Then the PMN increased rapidly and a normal differential count was restored between the 7th and 11th days after the first HN_2 injection. No corneal opacity was observed in these rabbits throughout the experiment.

Suppression of virus-induced corneal opacity by pretreatment of rabbits with intravenous injections of HN_2. Rabbits were given 2 intravenous injections of HN_2 in the same manner as in the preliminary experiment. Two days after the last injection of HN_2, when the number of leucocytes reached the lowest levels, both eyes were inoculated with 0.2 ml of NDV (4×10^8 PFU). As a control, normal rabbits were simultaneously inoculated with the same amount of virus into their anterior chambers. Frequency of corneal opacity, appearing 24 hours after virus injection, was compared between these two groups. Results are summarized in Table I.

In all experiments, the number of eyes showing corneal opacity was far smaller in HN_2-treated rabbits than in normal controls. Whereas the percentage of corneas showing opacity in HN_2-treated animals in any single experiment ranged from 0 to 33%, it ranged from 63 to 100% in untreated rabbits. In all, only 5 of 42 eyes of the HN_2-treated group showed corneal opacity whereas in the control group, 35 of 41 eyes showed the opaque corneas. It was noted that aqueous humor of these 5 opaque corneas of HN_2-treated rabbits contained few or no leucocytes.

Limited experiments were carried out to explore the influence of time of injection on the suppressive effect of HN_2 on the toxic reaction to NDV. A suppressive effect was observed irregularly in the rabbits pre-treated with 2 injections of HN_2 at a 48 hour interval and given an intraocular injection of NDV

immediately after the second injection of HN_2. Suppression of corneal opacity was not apparent in the animals which were given intraocular injections of virus immediately, 2 days or 4 days after a single injection of HN_2. It appears that treatment of animals with 2 injections of HN_2 before virus inoculation was an important factor in suppression of lesions.

Effects of pretreatment of rabbits with HN_2 on development of microscopic lesions of corneal endothelium and on virus titer in aqueous humor following intraocular injection of NDV. Ten rabbits were given 2 intravenous injections of HN_2 in the same manner as in the preliminary experiment. On the second day after the last injection of HN_2, 0.2 ml of NDV in allantoic fluid (4×10^8 PFU) was inoculated into both eyes. The same number of normal rabbits served as controls and virus was similarly inoculated. At each interval, 2 rabbits were taken from each group; degree of corneal opacity was checked and animals were immediately sacrificed by an intravenous injection of sodium Nembutal. Aqueous humor, for leucocyte count and virus assay, was aspirated from each eye with a separate tuberculin syringe. Extent of fusion was scored and total number of endothelial rosettes appearing on the entire endothelium was determined. Results are shown in Table II.

Development of corneal opacity was completely suppressed in all HN_2-treated animals of the 12 and 24 hour-groups. Fusions were less extensive than in corresponding control animals. On the other hand, a large number of rosettes still appeared on the corneas of the HN_2-treated rabbits. The number of leucocytes in aqueous humor was much smaller in HN_2-treated animals than in untreated animals. Titer of residual virus in aqueous humor of HN_2-treated animals was not different from that of controls, even though there was a marked suppression on development of corneal opacity in treated animals. These results indicated that suppression of corneal opacity in HN_2-treated animals was probably not due to rapid inactivation of virus in aqueous humor.

From the above results it is reasonable to assume that suppression of corneal opacity and fusion appears to be correlated with, but not necessarily due to, suppression of leucocytes, particularly the PMN, in aqueous humor. However, it is important to note that one rabbit in the 24 hour-control group showed no corneal opacity even though a large number of leucocytes was present in aqueous humor.

Relationship between persistent suppression on production of corneal lesions by NDV and number of leucocytes in aqueous humor following recovery from HN_2-induced leucopenia. If presence of leucocytes, particularly PMN, in aqueous humor is directly related to occurrence of endothelial fusion and corneal opacity, it is reasonable to postulate that when the number of leucocytes in the blood of HN_2-treated animals returned to normal level, intraocular inoculation of virus should cause a large number of PMN to appear in aqueous humor, and corneal opacity and fusion might be expected to develop as extensively as in control rabbits. To examine this possibility experiments were performed as follows:

Five rabbits were injected twice with HN_2 in the standard manner. Two days after the last injection of HN_2 blood leucocyte and differential counts of these animals were determined. At the same time 4 normal rabbits were allocated as controls, and blood leucocyte and differential counts were similarly carried out on these animals. Two-tenths ml of NDV in allantoic fluid (4×10^8 PFU) was inoculated into the anterior chamber of the right eye of each animal in both groups. The uninoculated left eyes served as controls. Twenty-four hours after virus injection, corneal opacity was recorded and 0.1 ml of aqueous humor was aspirated from the injected eye for leucocyte count. On the 13th or 20th day after virus inoculation, blood leucocyte and differential counts were again carried out on these groups of animals and NDV (4×10^8 PFU) was then inoculated into the anterior chambers of both eyes. Twenty-four hours after the second virus injection, corneas of these animals were checked for de-

gree of opacity, and at the same time 0.1 ml of aqueous humor was aspirated to determine total number of leucocytes in a drop. The results are summarized in Table III.

Typical corneal opacity developed after the second virus injection in all 4 control rabbits. However, rabbits treated with HN_2 did not develop corneal opacity even after the blood leucocyte count reached or exceeded a normal value and after leucocytes were present in large number in aqueous humor of these eyes. It was interesting to find that the suppressive effect of HN_2 on development of corneal opacity in response to NDV lasted for at least 13 and 20 days after the first NDV injection. It was considered

TABLE II. Effects of Pretreatment of Rabbits with HN_2 on Production of Microscopic Lesions of Corneal Endothelium and on Viral Titer in Aqueous Humor Following Intraocular Injection of NDV.

Hr after virus inoc.	Group	Rabbit No.	Eye	Corneal opacity	Microscopic changes: Fusion	Microscopic changes: Rosette	WBC/drop of aq.h.*	NDV in aq.h.* (PFU/ml)
1½	HN_2†	1	R	—	±	97	0	5.1×10^6
			L	—	±	96	0	5.9×10^5
		2	R	—	±	15	1	1.5×10^6
			L	—	±	16	0	1.2×10^6
	Control	1	R	—	ND	ND	7	4.8×10^6
			L	—	±	79	19	7.5×10^5
		2	R	—	±	110	5	2.9×10^6
			L	—	±	172	1	7.0×10^6
3	HN_2†	1	R	—	±	188	6	5.4×10^5
			L	—	±	110	0	6.4×10^6
		2	R	—	±	132	0	2.2×10^5
			L	—	±	151	0	8.3×10^5
	Control	1	R	—	±	297	974	5.4×10^5
			L	—	±	337	470	ND
		2	R	—	±	191	290	3.0×10^4
			L	—	±	120	540	7.0×10^5
6	HN_2†	1	R	—	+	‡	1	5.4×10^6
			L	—	+	‡	4	2.3×10^5
		2	R	—	± to +	‡	3	2.9×10^4
			L	—	± to +	‡	8	7.3×10^5
	Control	1	R	±	++	‡	610	1.9×10^5
			L	±	++	‡	850	3.0×10^5
		2	R	±	+	‡	534	1.7×10^5
			L	±	++	‡	2216	1.0×10^5
12	HN_2†	1	R	±	++	‡	71	4.0×10^4
			L	±	+	‡	131	4.0×10^4
		2	R	—	+	‡	6	5.8×10^4
			L	—	+	‡	8	5.2×10^3
	Control	1	R	++	+++	‡	241	2.6×10^4
			L	+	++	‡	970	2.1×10^4
		2	R	++	+++	‡	641	9.0×10^4
			L	++	+++	‡	968	2.0×10^4
24	HN_2†	1	R	—	± to +	‡	31	1.1×10^3
			L	—	± to +	‡	4	4.0×10^3
		2	R	—	+	‡	21	1.3×10^2
			L	—	+	‡	4	5.0×10^2
	Control	1	R	—	+	‡	1621	3.8×10^2
			L	—	+	‡	1221	9.5×10^2
		2	R	+++	+++	‡	1451	1.8×10^3
			L	+++	+++	‡	3360	6.2×10^2

* Aqueous humor (aq.h.).
† Two intrav. injections of HN_2 were given 2 and 4 days before intraocular inj. of NDV.
‡ Not suitable to count rosettes due to extensive fusions.
ND = Not done.

TABLE III. Persistent Suppression of Corneal Toxic Reaction to NDV after Termination of Leucopenia Induced by HN_2.

		1st intraocular inj. of NDV						2nd intraocular inj. of NDV					
Group	Rabbit No.	Blood WBC WBC/mm³	Blood WBC PMN (%)	Eye	Inoc. with NDV	Opacity after 24 hr	WBC/drop of aq.h.*	Days after 1st NDV inoc.	Blood WBC WBC/mm³	Blood WBC PMN (%)	Inoc. with NDV	Opacity after 24 hr	WBC/drop of aq.h.*
HN_2 treated‡	1	1825	1	R	Yes	—	31	20	7650	46	Yes	—	930
				L	No		ND				,,	—	1160
	2	950	0	R	Yes	±	6	20	10250	47	,,	+	730
				L	No		ND				,,	—	1500
	3	300	5	R	Yes	—	10	13	4950	40	,,	±	1560
				L	No		ND				,,	—	2160
	4	525	3	R	Yes	—	1	13	6150	42	,,	±	900
				L	No		ND				,,	—	916
	5	525	1	R	Yes	+	4	13	4250	39	,,	±	761
				L	No		ND				,,	—	819
Control	1	4250	39	R	Yes	+++	1714	20	ND	ND	Not		ND
				L	No		ND				Yes	++	2601
	2	6000	47	R	Yes	+++	2160	20	,,	,,	Not		ND
				L	No		ND				Yes	+++	1900
	3	5100	34	R	Yes	+++	960	13	,,	,,	Not		ND
				L	No		ND				Yes	+++	3161
	4	8800	40	R	Yes	+++	1500	13	,,	,,	Not		ND
				L	No		ND				Yes	+++	1916

* Aqueous humor (aq.h.) was removed at the time when corneal opacity was scored.

† Corneal opacity produced by the first inj. of NDV still persisted.

‡ Two intrav. injections of HN_2 were given 2 and 4 days before the first intraocular inj. of NDV.

ND = Not done.

possible that this late suppressive effect was due to formation of antibodies against NDV. However it was found that there was no significant antibody level against NDV in these animals at the time of second injection of virus. It may be possible that leucocytes at 13 and 20 days after HN_2-treatment are functionally inactive in formation of corneal opacity by NDV.

Discussion. In rabbits pretreated with 2 intravenous injections of HN_2, corneal opacity as well as endothelial fusion was suppressed while rosettes appeared as extensively as in controls. It was reported previously(2) that in the *in vitro* system injection of NDV into the anterior chamber caused formation of numerous rosettes but did not induce either opacity of the cornea or an appreciable degree of fusion of endothelial cells. This experimental evidence suggests that corneal opacity in virus-induced toxic reaction is more closely associated with endothelial fusions than with endothelial rosettes.

Mechanisms by which virus produced these lesions are not understood. However, the present study appears to indicate that leucocytes are not essential for production of a toxic reaction by NDV in the rabbit cornea. This is supported by the following observations: 1) Following intraocular inoculation of NDV, rabbits pretreated with HN_2 occasionally developed opaque corneas. These eyes showed few or no leucocytes in their aqueous humor. 2) Eyes of normal rabbits inoculated with a toxic amount of NDV sometimes failed to produce an opaque cornea and fusions even though there was a large number of leucocytes in their aqueous humor. 3) In the HN_2-treated rabbits, suppressive effect of HN_2-treatment on the development of corneal opacity lasted for at least 13 and 20 days after treatment. This long lasting suppression was observed in eyes which contained numerous leucocytes in aqueous humor. It appears that infiltration of leucocytes into ocular tissues and aqueous humor is secondary to changes induced by the virus in the eye. The role of leucocytes in the toxic cornea is unknown at present.

It has been reported that mustard gas inactivates NDV *in vitro*(5). However titer of residual virus in aqueous humor of eyes of HN_2-treated animals was as high as that of controls at any time. Therefore, suppression of virus-induced corneal toxicity by pretreatment with HN_2 is not due to rapid inactivation of virus in aqueous humor.

Intravenous injection of HN_2 simultaneously with intraocular inoculation of virus failed to suppress development of corneal opacity. It appears that pretreatment of rabbits with HN_2 is an essential factor in suppression of virus-induced corneal opacity. These findings suggest that HN_2 modifies the host's interaction with virus. In virus-induced corneal reaction, however, it is not known whether HN_2 exerts its suppressive action on corneal endothelium, on tissues other than corneal endothelium, or on humoral factors which may play an essential role in the pathogenesis of NDV. It was suggested that in the Shwartzman phenomenon HN_2 exerts its suppressive action on the reticuloendothelial system, primarily the vascular endothelium(6). Possibly a similar modification of corneal endothelium is brought about by HN_2 injection to render host tissue less susceptible to virus activity. Further studies are necessary to make a proper evaluation of this possibility. This attitude is also important in studying the suppressive action of HN_2 on the Shwartzman phenomenon, especially when considering the suggestion of Stetson and Good(7) that inhibition of the Shwartzman phenomenon can be correlated with leucopenia induced by HN_2.

Rose and Gellhorn(8) failed to modify influenza virus-induced pulmonary lesions in mice by pretreating the animals with daily intraperitoneal injections of HN_2 beginning 2 days before or immediately after virus inoculation. However, in view of the suppressive action of HN_2-treatment on NDV-induced toxic reaction, it is desirable that HN_2 should be further explored for its possible effectiveness in preventing lesions in other tissues infected by NDV or other viruses known to produce a similar toxic effect in animals.

Summary. Pretreatment of rabbits with 2 intravenous injections of nitrogen mustard (HN_2) partially suppressed production of

NDV-induced toxic corneal reactions. In most animals this treatment suppressed development of opacity and reduced markedly the extent of fusions of endothelial cells. It did not inhibit formation of endothelial rosettes. The suppressive effects in the HN_2-treated animals lasted for at least 20 days, well after leucopenia had disappeared. Decreased response to injected virus is neither a result of suppression of circulating leucocytes nor of rapid inactivation of virus in aqueous humor.

Treatment of rabbits with a single intravenous injection of HN_2 4 days, 2 days, or immediately before virus injection caused no demonstrable suppression of virus-induced toxic reactions of rabbit corneas. Leucocytes appeared to play no essential role in production of corneal reaction.

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Correlation of Pituitary Ribonucleic Acid Levels and ACTH Production Following Adrenalectomy and Cortisone Administration.* (26357)

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It has been inferred that withdrawal of adrenal cortcial hormones enhances both release and synthesis of pituitary adrenocorticotrophic hormone (ACTH), but with a predominent effect on synthesis. The latter mechanism serves to explain the increased concentration of ACTH in pituitary tissue following adrenalectomy(1,2,3). The inhibitory effect of adrenal cortical hormones on ACTH content of pituitaries and plasma likewise has been attributed to alteration of rates of trophic hormone production and release (3,4).

Since the relationship of ribonucleic acid (RNA) and protein synthesis has been shown to be applicable to pituitary hormone production following adrenalectomy(5), one can correlate fluctuations in tissue content of ACTH following adrenalectomy and cortical hormone administration, as demonstrated by Fortier(1,4), with RNA levels in the hypophysis. This report, based on pituitary RNA content following adrenalectomy or adrenal cortical hormone treatment, is an attempt to correlate protein synthetic activity with conent of trophic hormone in the tissue.

Methods. Male rats of the Holtzman strain were bilaterally adrenalectomized *via* dorsal approach and maintained on Purina Laboratory Chow pellets and 1% NaCl *ad lib.* Unoperated rats of the same age, receiving tap water in place of salt solution, served as controls. At intervals of time from ½ hour to daily and weekly post-operative periods (Table I), adrenalectomized and control rats were sacrificed. Other rats received

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TABLE I. Effect of Adrenalectomy on Pituitary RNA.

Post-operative time	μg RNA/mg tissue	Post-operative time	μg RNA/mg tissue
Control	7.5 ± .3*(33)		
½ hr	9.9† ± .2 (6)	30 hr	7.9 ± .2 (15)
1 "	8.4 ± .1 (6)	48 "	12.6† ± .5 (9)
2 "	7.6 ± .4 (6)	7 days	8.0 ± .3 (12)
3 "	8.0 ± .1 (6)	14 "	9.5† ± .2 (15)
6 "	8.7 ± .2 (9)	21 "	8.4 ± .5 (18)
12 "	7.0 ± .3 (24)	32 "	9.4† ± .5 (6)
24 "	6.9 ± .3 (12)		

* Stand. error.
† Difference from controls statistically significant.
No. of animals in parentheses.

Cortone Acetate (Cortisone Acetate, Merck), 6 mg/100 g I.P., or an equal volume of saline daily for 4 weeks. At time of sacrifice, rats were decapitated, anterior pituitary tissue collected, weighed and assayed for RNA content as previously reported(6).

Results. During the first 30 hours following adrenalectomy, the only significant rise in pituitary RNA over control levels, of various time periods studied, occurred in those rats sacrificed at ½ hour post-operatively. A rise of 32% decreased to values not significantly different from controls at one, 2, 3 and 6-hour intervals (Table I). Concentration of pituitary RNA at 12 and 24-hour post-operative periods was somewhat below that of controls, but returned to a normal level 6 hours later (Table I). The most marked increase in RNA concentration, a 68% rise, occurred in those rats adrenalectomized for 48 hours. Pituitary RNA of rats operated for 7, 14, 21 and 32 days fluctuated from normal to elevated levels rhythmically (Table I).

The dosage of cortisone utilized produced marked reduction in body and adrenal weights of treated animals compared to controls. The treatment also resulted in a 21% reduction in concentration of pituitary RNA (Table II).

Discussion. Plotting pituitary RNA in μg/mg tissue against time after adrenalectomy, the curve in Fig. 1 is obtained. Comparison of this curve with that depicted for pituitary ACTH concentration after adrenalectomy(1) shows remarkable similarity up to 2 post-operative days. The rise in pituitary RNA ½ hour post-operatively coincides with elevated tissue content of ACTH at that time. Furthermore, the fall in ACTH

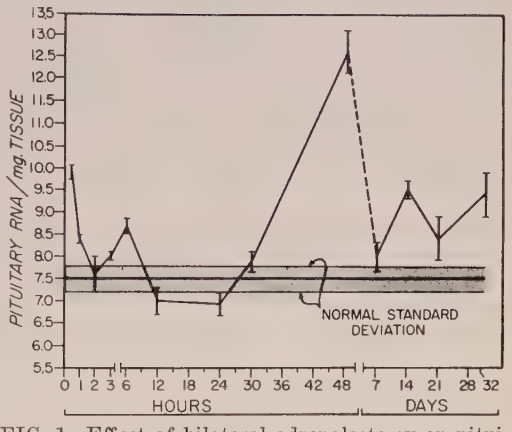

FIG. 1. Effect of bilateral adrenalectomy on pituitary RNA concentration (in μg/mg tissue) in relation to time (hr and days). Shaded area indicates control value with stand. error (S.E.). Lines above and below each of the points on the curve indicate S.E.'s.

concentration to below control levels during the next 24 hours is reflected in the pituitary RNA by a return to normal at 6 hours and subnormal levels at 12 and 24 hours post-adrenalectomy. The second rise in pituitary ACTH begins between the first and fourth post-operative day in Fortier's curve(1), which once again coincides with the rise in pituitary RNA 48 hours after surgery. How-

TABLE II. Effect of Cortisone on Pituitary RNA.

Group	Body wt (g)	Rel. adr. wt (mg/100 g B.W.)	Pit. RNA (μg/mg tissue)
Controls (20)—.2 cc saline/100 g/day	259 ± 5*	12.7 ± .3*	8.0 ± .2*
Treated (19)—6 mg cortisone/100 g/day	170† ± 6	7.9† ± .4	6.3† ± .2

* Stand. error.
† Difference from controls statistically significant ($p < .001$).
No. of animals in parentheses.

ever, continued increase in hormone concentration in the tissue is not reflected in sustained high levels of pituitary RNA.

The polyphasic response of pituitary ACTH to adrenalectomy can be explained in relation to protein synthetic activity taking place in the tissue as measured by RNA content. The initial rise in RNA ½ hour postoperatively, indicating accelerated rate of synthesis, accounts for increased tissue content of ACTH. The subsequent decrease in tissue content of ACTH, reflecting predominance of hormone release over synthesis(1), is revealed by the diminished RNA values from one to 30 hours after surgery.

The secondary rise in ACTH content is preceded by markedly accelerated rates of protein synthesis as evidenced by high levels of pituitary RNA. The fact that the tissue continues to accumulate increased amounts of ACTH without accompanying indices of accelerated synthesis may be explained by one or both of the following hypotheses. It may be that once initiated (48 hours postadrenalectomy), the pituitary gland can produce increased amounts of ACTH without an exaggerated rate of protein synthesis. Or it may be that the accumulation of endogenous ACTH in the tissue exerts a depressing effect on the synthetic processes comparable to the observation made by Gemzell and Heijkenskjöld utilizing exogenous ACTH to suppress pituitary content of the hormone in adrenalectomized animals(7).

The depressing effects of adrenal cortical steroids on pituitary ACTH content(3,4) are likewise reflected in the RNA picture. If cortical hormones act to inhibit ACTH synthesis, as suggested by Hodges and Vernikos (3) and Fortier(4), the low level of tissue RNA following cortisone treatment adds further support to the pituitary protein synthetic inhibition produced by adrenal steroids.

That the tissue content of RNA is associated with protein synthetic activity and not hormone release is based on the following observations. Although pituitary RNA and ACTH levels are high within a few days following adrenalectomy, circulating adrenocorticotrophin cannot be easily detected until 3-5 weeks after surgery(8). In cortisone-treated rats, the small adrenal weights together with reduced pituitary RNA and ACTH contents may indicate inhibition of both synthesis and release of trophic hormone. However, the failure to completely prevent the rise in blood corticotrophin of stressed-adrenalectomized rats with adrenal steroid(3) indicates the action of cortical hormone is not on release.

Summary. The concentration of RNA in pituitary tissue was determined following various periods of adrenalectomy and prolonged cortisone treatment. Elevations of pituitary RNA at ½ and 48 hours post-operatively correlate with increased tissue content of ACTH reported for the post-adrenalectomy periods. Reduction in level of tissue RNA following cortisone administration likewise correlates with decreased amounts of ACTH in pituitaries reported after such treatment. The data, utilizing tissue RNA as an index of protein hormone synthetic activity, provide further information that indicate adrenal cortical steroids regulate synthesis of ACTH.

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Effect of Friend Virus in Swiss and DBA/1 Mice on Fe^{59} Uptake.* (26358)

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Friend Virus Disease is typically associated with malignant reticulum cell proliferation and pronounced erythroblastosis in adult mice of Swiss, DBA/2(1-3) and DBA/1 strains. Grossly, the most striking feature is the associated progressive hepatosplenomegaly. Latent period in susceptible mice is relatively short after inoculation of virus.

The erythroblastosis which was regularly observed prompted us to undertake a study of some of the features of erythropoiesis in the disease. The 24 hour uptake of Fe^{59} was one of the parameters selected for study, because of its marked sensitivity as an index of erythropoiesis(4,5). This report describes a marked peripheral erythroblastosis and erythrocytosis which parallels spleen involvement in the disease and the marked selective uptake of Fe^{59} by the spleen, beginning very early in the disease.

Materials and methods. Mice employed in this study were HA/ICR Swiss and DBA/1 mice 6-8 weeks old, obtained from our own breeding colonies and maintained on a diet of Derwood-Morris pellets.

Friend Virus was obtained through the courtesy of Dr. Charlotte Friend, Sloan-Kettering Inst., New York City. The disease was maintained by both cellular and cell-free passages of infected spleens. The resultant disease was the same regardless of type of inoculum used. At the beginning of our study, the virus was in its eighth serial passage in this laboratory and percentage of "takes" with a 10^{-1} inoculum in our mice at this passage was 85-100%. A pool of virus was obtained by harvesting a large number of infected spleens. A 10% fine mince suspension of the infected spleens in cold Locke-Ringer solution was homogenized in a Ten Broeck grinder and centrifuged for 10 minutes at 5000 G in a refrigerated centrifuge. A broth culture of *E. coli* was added to the supernatant prior to filtration through a HA-grade millipore filter. Filtrate was cultured in broth to check impermeability of the filter to *E. coli.* Pooled filtrate was stored in sealed ampoules in a dry ice chest at –70°C. Dilutions ranging from 10^{-1} to 10^{-6} were made of the pooled filtrate. Two-tenths of a milliliter of the dilutions from 10^{-1} to 10^{-6} were injected intraperitoneally into mice. A portion of the mice were sacrificed at regular intervals for studies described below. A minimum of 15 mice were used to establish each point in Fig. 1-4 and for each average figure in Tables I, II, III.

Twenty-four hours prior to sacrifice, one μc of Fe^{59} in 0.5 ml of unbuffered 0.9% NaCl solution was injected into the jugular vein of each mouse. At time of sacrifice, mice were bled from aorta and various organs removed and weighed. Radioactivity in blood and organs was measured in a Nancy-Wood well-type scintillation counter. Radioactivity in an aliquot of the original Fe^{59} solution used for injections was similarly measured. Uptake of Fe^{59} in the blood and organs was calculated by a previously described method(6).

White cell and rbc counts were performed on blood from the aorta with the use of standard human diluting pipettes and hemocytometers. Blood films were stained with Wright-Giemsa Stain. Hematocrits were done on aorta blood with Drummond microhematocrit capillary tubes.

Results. Tables I and II show 2 striking features of Friend Virus Disease in Swiss and DBA/1 mice. First, there is a progressive rise in hematocrit throughout the course of the disease. Secondly, there is a marked increase in 24 hour uptake of Fe^{59} by the spleen. This is particularly striking at 7 days post infection when there is greater than a 5-fold increase in spleen uptake as

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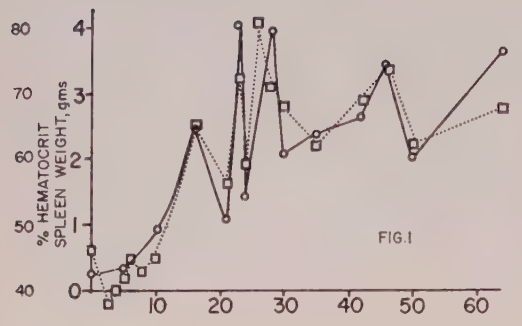

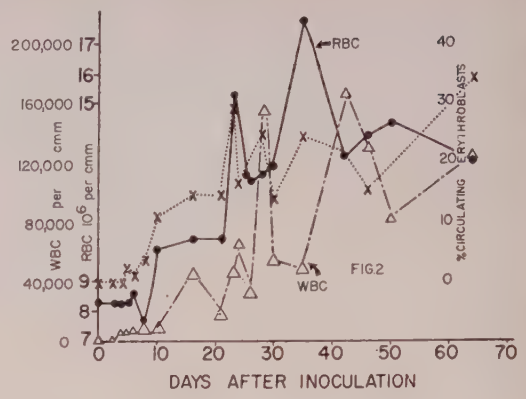

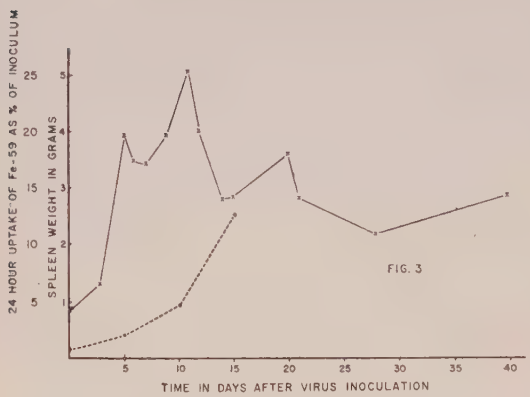

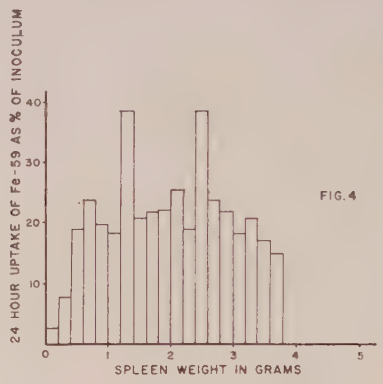

FIG. 1. Increase of spleen wt ○, and % hematocrit □ in Swiss mice infected with Friend Virus (10^{-1}).

FIG. 2. Changes in circulating blood cells in Swiss mice with time after infection with Friend Virus. ●, RBC; △, WBC count; and ×, % circulating erythroblasts, same mice as in Fig. 1.

FIG. 3. Mass of spleen, ⊙, and 24 hr uptake of Fe^{59}, ×, in Swiss mice infected with Friend Virus (10^{-1}).

FIG. 4. 24 hr uptake of Fe^{59} in spleens of Swiss mice infected with Friend Virus (10^{-1}) as a function of the spleen wt in g.

compared to uninfected controls. This is especially interesting because the increase in Fe^{59} uptake by the spleen precedes the onset of signs of the characteristic disease syndrome. In contrast to selective uptake of Fe^{59} by the spleen, other organs show no significant increase in uptake as compared to the controls.

Figures shown in Table I and II under controls represent values obtained at 7 days. Control measurements were also done at 14, 21, 28, 35 and 40 days. However, since values obtained on those days were essentially the same as those obtained at 7 days they were not included in this table.

The hematocrit was slightly below normal at 7 days (Tables I and II). This led to a more detailed study of post-infection hematocrit in Swiss and DBA/1 mice. Since Swiss and DBA/1 mice responded similarly to Friend Virus, subsequent data will pertain only to Swiss mice. Fig. 1 shows the hematocrit pattern following infection. Hematocrit levels dropped immediately post-infection, reached minimal level at about 3 days, then rose sharply. There was a close parallel between the rise in hematocrit and splenic weight. At 16 days average spleen weight was about 20 times that of uninoculated control animals.

Accompanying the rise in hematocrit and increase in splenic weight were changes in circulating blood elements. Fig. 2 demonstrates the progressive increase in numbers

TABLE I. 24-Hour Uptake* of Fe^{59} and Hematocrit ICR/HA Swiss Mice at Weekly Intervals Following Inoculation with Friend Virus (10^{-1}).

	7 days	14 days	21 days	28 days	Controls†
Blood	21.1 ± 5.4	28.1 ± 3.4	25.3 ± 6.3	25.9 ± 4.5	26.9 ± 3.7
Liver	18.6 ± 3.9	13.0 ± 6.4	8.1 ± 1.8	10.8 ± 5.0	11.3 ± 2.9
Spleen	17.6 ± 2.8	12.3 ± 4.4	13.8 ± 5.8	11.1 ± 1.5	3.0 ± .67
Kidney	1.0 ± .9	1.1 ± .3	.9 ± .22	1.1 ± .2	.8 ± .2
Femur	.11± .4	.11± .1	.15± .13	.2 ± .1	.3 ± .16
% hematocrit	39.5 ± 3.3	57.4 ±11.3	63.6 ±11.8	71.1 ± 8.1	43.5 ± 1.9

* Uptake in 24 hr expressed as % of inoculum.
† Controls at 7 days are shown. These are essentially identical with those at subsequent times.

TABLE II. 24-Hour Uptake* of Fe^{59} and Hematocrit in DBA/1 Mice at Weekly Intervals Following Inoculation with Friend Virus (10^{-1}).

	7 days	14 days	21 days	28 days	Controls†
Blood	24 ± 6.1	26.1 ± 2.1	25.9 ± 1.2	25.1 ± 6.2	27.2 ± 1.4
Liver	14.5 ± 2.1	15.1 ± 1.8	12.1 ± 2.4	15 ± 1.3	13.4 ± 1.2
Spleen	18.5 ± 3.4	14.2 ± 1.2	14.8 ± 1.8	12.8 ± 4.3	2.8 ± .54
Kidney	1.2 ± .8	1.1 ± .5	1.1 ± .13	.9 ± .23	1.2 ± .8
Femur	.11± .05	.13± .08	.14± .09	.18± .09	.3 ± .09
% hematocrit	37.8 ± 1.2	62.3 ± 8.5	60.3 ± 9.5	74.8 ± 7.2	41.5 ± 1.2

* Uptake in 24 hr expressed as % of inoculation.
† Controls at 7 days are shown. These are essentially identical with those at subsequent time.

TABLE III. Spleen Weight and Fe^{59} Uptake of Spleen and Hematocrit at 14 Days after Friend Virus Inoculation in Swiss Mice at Various Dilutions of Virus.

	Inoculum, dilution of virus filtrate				Normal spleen filtrate†	No filtrate inoculated
	10^{-1}	10^{-2}	10^{-3}	10^{-6}§	10^{-1}	—
Spleen wt (g)	1.83 ± .9	.35 ± .02	.27 ± .04	.14 ± .02	.14 ± .03	.17 ± .04
Fe^{59} uptake,* %	16.0 ±3.6	7.6 ±2.6	5.9 ±2.3	2.6 ± .6	2.7 ± .04	2.3 ± .3
Hematocrit, %	51	44	42	41	42	41
Fe^{59} uptake/g‡	9.9 ±3.0	24.1 ±7.9	23.4 ±5.5	18.7 ±4.3	20 ±3.5	14.9 ±4.3

* 24-hr uptake of Fe^{59} of whole spleen as % of inoculum.
† Normal spleen filtrate without virus.
‡ 24-hr uptake of Fe^{59} in spleen expressed as % of inoculum/g spleen.
§ 10^{-4} and 10^{-5} not included; data similar to that seen for 10^{-6}.

of circulating leucocytes, primarily granulocytes, erythrocytes and erythroblasts. The rise in erythroblasts to 34% on the 64th day led to additional studies designed to assess the role of the spleen in the increased erythropoiesis. Fig. 3 shows 24 hour Fe^{59} uptake in the spleen as related to spleen size. This demonstrates that the increase in Fe^{59} uptake begins very early and precedes splenic enlargement. Fe^{59} uptake levels off at about the time the spleen reaches its maximum size (about 2.5 g). Table III demonstrates in more detail the correlation between splenic weight, Fe^{59} uptake and hematocrit. While total uptake of Fe^{59} by the spleen is greater with the more advanced disease and larger splenic mass, uptake on a per gram basis decreases from earlier levels and falls to a point well below control levels.

Discussion. These studies demonstrate that Friend Virus produces, soon after infection, a remarkable increase in selective uptake of Fe^{59} by the spleen, accompanied by an increased splenic mass, an increase in circulating leucocytes and an increase in hematocrit characterized by a marked increase in circulating erythroblasts and erythrocytes. While there is an increase in number of white and red cells, a rise in platelets was not evident in peripheral blood.

Repeated histologic examination of bone marrow of infected animals at various stages

of the disease consistently revealed no hyperplasia. This observation, coupled with the findings described above, points strongly to the spleen as the main source of the erythroblastosis, increased red cell count, and hematocrit in peripheral blood. The very early rise of Fe^{59} uptake by the spleen suggests that the virus may exert a direct stimulating effect on erythroid elements in the spleen. Continued stimulation results eventually in a markedly increased hematocrit with many immature erythrocytes in peripheral blood. Increase in leucocytes, without evidence of increased medullary activity, suggests that the myeloid elements of the spleen may also be stimulated.

As the disease progresses there is decreased uptake of Fe^{59} by the spleen on a weight basis. This may be due to malignant proliferation of reticulum cells in the spleen so that the ratio of blood forming elements to reticulum cells diminishes progressively with advancing disease.

Unlike Polyoma Virus(8,9,10), the Friend Virus is apparently strain specific(1,2,3,7). Adult mice of Swiss and DBA/2 strains are highly susceptible whereas mice of PRI, C_{57} Bl/6, A, C_3H and ($C_{58} \times$ BALB/c) F_1 strains are nonreactive. Our data indicate that DBA/1 strain is susceptible to the Friend Virus, to the same magnitude as HA/ICR Swiss mice.

Summary. 1. These studies demonstrate that Friend Virus produces in HA/ICR Swiss and DBA/1 mice very soon after infection, a marked increase in selective uptake of Fe^{59} by the spleen. This selective uptake is not found in other tissues in Friend Virus Disease. 2. Friend Virus Disease characteristically produces a malignant proliferation of splenic reticulum cells, but is further characterized by a progressive elevation of hematocrit and rbc count which is related to splenic involvement in the disease. 3. The DBA/1 strain is shown to be susceptible to Friend Virus to the same magnitude as HA/ICR Swiss mice.

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Thymectomy and Thymic Grafts in Mouse Viral Leukemia.*† (26359)

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Thymectomy has been found to decrease greatly the incidence of spontaneous leukemia in AK(1) and C58 mice(2) and that induced by whole body irradiation in C57B1 mice(3), by administration of methylcholanthrene to DBA mice(4), or by neonatal injection of leukemia virus in C3H or AK mice(5,6,7).

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† This work was done in collaboration with Dr. Jacob Furth.

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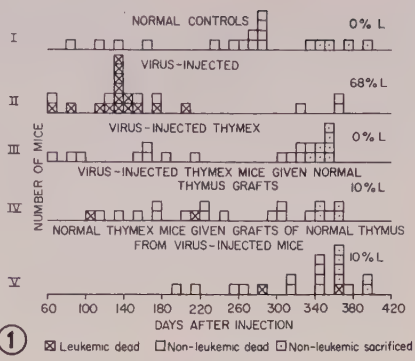
The following studies were carried out to determine the influence of thymic grafts from normal donors in restoring capacity of virus-injected, thymectomized mice to develop leukemia and to determine the origin of the leukemic cell under such conditions.

Materials and Methods. Virus. Passage virus, Agent A of Gross, has been carried in our laboratory in 7 serial passages since 1957 (6). Preparation and storage of filtrates has been described. *Mice.* C3H/f/Bi/G/L,§ hereafter designated Z mice, were used in the first experiment, and hybrids of this line with AKR/Jax in the second. Males and females were caged together. Litters from virus-injected females were discarded; those from uninjected controls were raised. *Injection of virus* to newborn mice (less than 1 week of age) in 0.025-0.07 cc amounts was done intraperitoneally using a No. 30 needle. *Thymectomy* was performed under nembutal-ether anesthesia at 4 to 8 weeks of age by incising the sternum and removing the thymic lobes with curved iridectomy forceps. Mice developing thymic tumors due to incomplete thymectomy were excluded from experimental groups. *Intercurrent infections* were treated by addition of veterinary terramycin to drinking water. *Fighting* among AKR males used in bioassays of induced leukemias was diminished by addition to drinking water of a suspension of meprobamate (Miltown), 100-200 mg/liter. *Thymic grafts* (one lobe of thymus from a healthy 5 to 8 week old donor) were implanted subcutaneously in the axillary region. *Bioassays* of leukemias were made by injecting intramuscularly 0.1 cc of a 10% suspension of minced tissue (5-13 million cells), unless otherwise indicated. *Sections* were taken from most leukemic and many non-leukemic animals with special attention to thymectomy sites. The latter was removed *en bloc* and sectioned longitudinally. Kidney and thyroid were routinely examined for polyoma lesions.

Results. Thymus grafts in thymectomized virus-injected Z mice. The experimental groups are indicated in Fig. 1. None of 18 normal controls developed leukemia (Group I). Of 22 virus-injected intact mice, 15 (68%) developed leukemia with an average latent period of 135 days (Group II). Of 17 virus-injected mice thymectomized at 4 to 6 weeks of age none developed leukemia (Group III). Of 20 virus-injected mice thymectomized at 4 to 6 weeks of age and grafted with normal thymus (Group IV), 2 developed leukemia, one generalized with tumor in the thymic graft at 225 days, and the other generalized without gross tumor in the graft at 109 days. Of 19 uninjected thymectomized mice grafted with thymus from virus-injected mice (Group V), 2 developed leukemia with tumor in the grafts at 274 and 369 days.

Evidence for *persistence of virus in thymectomized virus-injected* non-leukemic mice is presented in Table I. Six infected thymuses removed at 38 days after injection (i.e., during the latent period of leukemia)



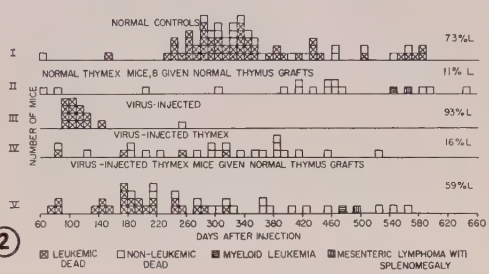


FIG. 1. Effects of grafts of normal thymus from virus-injected or uninjected mice on incidence of leukemia in normal and virus-injected thymectomized Z mice.

FIG. 2. Effects of normal AK and Z thymus grafts on incidence of leukemia in virus-injected and uninjected thymectomized AK/Z mice.

§ C3H mice, foster-nursed, originally obtained from Bittner, carried for many generations by Gross, and subsequently by Levinthal.

TABLE I. Viral Assay of Non-leukemic Virus-injected Thymectomized Z Mice by Means of Tissue Grafts on Normal Z and AK/Z Mice.

Tissue grafted	Mice assayed Age, days	No.	Recipients No.	Strain	Age	Leuk.	Latency, days
			Normal host (control)				
Lymph nodes	254	1	1	Z	newborn	0	
			7	AK/Z	"	1	365
			Virus-injected host				
Thymus	38	6 ind.*	6	Z	adult	2	200, 300
Spleen, liver, lymph nodes	273	3 pooled*	1	Z	newborn	0	
			4	AK/Z	"	4	167, 184, 214, 230
Idem	254	2 "	3	Z	"	0	
			2	AK/Z	"	1	142
"	240	2 "	2	Z	"	1	187
			2	AK/Z	"	2	145, 157
		Total	12	Z		3	187, 200, 300
			8	AK/Z		7	142–230 (177)

* Grafts were assayed individually or pooled.

and grafted individually on 6 intact young adult male Z mice produced 2 thymic lymphomas at 200 and 300 days without tumor at site of the graft, which suggests viral induction of leukemia.

Other tissues from virus-injected mice in which leukemia was prevented by thymectomy were assayed at 240 to 273 days in newborn Z and AK/Z mice. One of 6 Z, and 7 of 8 AK/Z mice developed leukemia. The short latent period in AK/Z mice compared with uninjected controls (Fig. 2) and the high per cent of leukemia indicate superinfection with leukemia virus. However, the latent period was long enough to exclude the likelihood of leukemia due to presence of a few leukemic cells in the assay material. Lymph nodes from an 8 month old uninjected Z mouse assayed as a control had no leukemogenic activity (Table I).

Persistence of virus in tissues of infected thymectomized animals that fail to develop leukemia is to be expected from observations with AK mice showing that non-leukemic parents (non-thymectomized (8) or thymectomized(1)) transmit the capacity to develop leukemia, as do leukemic parents. Also presence of virus in non-leukemic tissues of AKR embryos was demonstrated by Gross(9).

Thymus grafts in thymectomized virus-injected AK/Z mice. Experimental groups are indicated in Fig. 2. Incidence of spontaneous leukemia in normal AK/Z mice was about 60% with a latent period of 10 to 12 months (Group I). Of 18 uninjected mice thymectomized at 6 to 8 weeks of age (8 were later grafted with normal AK and Z thymus), none developed lymphoid leukemia (Group II). Of 15 virus-injected intact mice, 14 (93%) developed leukemia with an average latency of 110 days (Group III). Of 25 virus-injected mice thymectomized at 6 to 8 weeks of age, 4 (16%) developed generalized leukemia (Group IV).

In Group V, 44 virus-injected thymectomized mice (22 males, 22 females) were grafted within a week after thymectomy with one lobe of normal AK and Z thymus.

The donors were about the same age and sex as recipients, with the following exceptions: 14 females were grafted from male donors, and received a second graft (2 to 4 weeks later) from female mice; 2 males received only one female thymus graft (one Z and one AK), and one female only 2 male grafts.

Of 44 mice of this group, 24 (55%) developed generalized lymphoid leukemia at less than one year of age. In 6 of these there was a large lymphoma at site of the Z graft, and in one at the site of the AK graft also. In 7 of the leukemias there was a large lymphoma at site of the AK graft only. In 3 there was a large tumor at site of the

TABLE II. Bioassay for Histocompatibility of Virus-induced and Spontaneous Leukemias in AK/Z, Z and AK Mice.

Leukemia assayed			Latency (days) of leukemia in recipients		
Mouse	No. of Cells assayed (approx.)	Passage	AK/Z	Z	AK
			Spontaneous AK		
1	10^7	I	16, 16, 16, 16	N N N N N*	16, 16, 16
2	10^7	I	27, 27, 27	N N N N	20, 23, 24, 24
3	10^5	I	89, N	N N N	N N N
			Virus-induced Z		
48	10^7	I		14, 14, 28, 32, 35	N N N
6	10^7	I		15, 15, 15, 18, 21	N N N
7, 24, 25†	10^7	I		18, 25, 26, 27, 28, 28, 28, 30	N N N N
31	2×10^5	I	31, 31		N N N
			Virus-induced AK/Z		
25	1.3×10^7	I	13, 15, 15	15, 19, 20, N N	13, 13, 15
	9×10^6	II	15, 16, 18	28, 41, N	15, 33, 34
	2.5×10^4	III	16, 17, 20	23, 23, 23	17, 20, 23
34	10^7	I	16, 16, 17	39, 39, N N N N	N N N
	5×10^5	II	21, 21, 21	38, 47, 54	N N N
18	4×10^6	I	22, 22, 22	50, 59, N N N	28, 28, 49
	9×10^5	II	23		28, 28, 40
5	5×10^4	I	14	N N N	N N N

* N = No growth. † Pooled.

AK and a small tumor at site of the Z graft. The remaining 8 leukemias were generalized; in some of these the small grafts, identified microscopically, were infiltrated with leukemic cells.

Myeloid leukemia is known to occur in thymectomized AK mice at late age(1), and Gross believes that it may be caused by the same virus which causes thymic lymphona (10). It is noteworthy, therefore, that in the present series, one thymectomized control (Group II, Fig. 2) and one virus-injected thymeqtomized thymus-grafted mouse (Group V, Fig. 2) developed myeloid (chloro-) leukemia at 548 and 476 days respectively.

Two late survivors, one thymectomized control (Group II, Fig. 2) and one virus-injected thymectomized thymus - grafted mouse (Group V, Fig. 2) had greatly enlarged mesenteric lymph nodes and splenomegaly at 569 and 490 days respectively. Earlier studies indicated that this is reticulum cell or lymphoid neoplasia, different from thymic lymphoma. In the present cases, microscopic studies to determine cell type were inconclusive because of autolysis.

Presence of polyoma in filtrates. In the first experiment, one of 22 virus-injected intact Z mice developed typical bilateral salivary gland carcinomas. The leukemia passage virus used in this experiment caused cytopathogenicity in embryo tissue cultures with formation of hemagglutinins. Supernatant fluids of these tissue cultures induced typical polyoma lesions in newborn AK/Z mice.

Immunogenetic character of leukemic tumors arising in thymic grafts. The question whether grafted normal thymus supplies a factor needed by the virus for leukemic transformation of normal non-thymic lymphocytes of the host calls for an immunogenetic characterization of the leukemias arising in thymectomized virus-injected F_1 mice grafted with normal thymus from the parental strains.

As a base line, the immunogenetic relationship of AK, Z and AK/Z leukemic cells was investigated (Table II). a) Spontaneous AK leukemias (using a heavy inoculum containing an estimated 10^7 cells) grew in AK and AK/Z and failed to grow in Z mice.

TABLE III. Summary of Transplantation Assays for Immunogenetic Character of Induced Leukemia and Tumors in Thymic Grafts.

AK/Z donor						
	Tumor in donor*		Transplantation pattern†			
No. assayed	Z	AK	AK	Z	AK/Z	No takes
7	0	+	2 (+1)	0	1 (+1)	2
5	+	0	1	1	2 (+1)	0
4	+	+	(1)	0	3	0
6‡	0	0	1 (+1)	0	2 (+1)	1

* Thymus graft site.

† Numbers in AK column indicate takes in AK and AK/Z; in Z column in Z and AK/Z; and in AK/Z column in AK/Z only. No. in parentheses indicate some uncertainty as to the transplantation pattern because few animals were used and only some took.

‡ Generalized leukemia with no tumor at graft site.

One leukemia tested at a lower inoculum grew only in AK/Z. No spontaneous Z leukemias were available for assay. During 4 years of study, only one spontaneous leukemia was encountered in Z mice. b) Viral induced leukemias in Z mice have uniformly grown in Z and AK/Z but not in AK mice. c) Viral induced leukemias in AK/Z mice always grew in AK/Z, but unexpectedly 3 of 4 grew also in Z, and 2 of those 3 also in AK mice.

The irregularities and failures of cell doses at or below 10^5 are disturbing. This may be due to inadequate inbreeding (homozygocity) of the strain, or to the character of the leukemic cells, or to some unsuspected technical error. Subcutaneous grafts, used here, are known to require more cells than intraperitoneal or intravenous grafts. These observations show a puzzling deviation from the classical histocompatibility pattern and impose limitations on interpretation of the results of grafting in the host systems under study.

Transplantation assays were performed with leukemic cells from 22 of the 24 thymectomized AK/Z mice which received grafts of AK and Z thymuses. The transplantation patterns are indicated in Table III. The donors fall into 4 groups according to presence or absence of lymphoma at site of the grafts as shown in columns 2 and 3.

There was no absolute conformity between the two indicators of cell type: tumor formation at graft site and transplantation pattern. Only one tumor was clearly Z type, as indicated by both tumor formation in the Z graft and transplantability in Z but not in AK mice. Two with AK transplantation pattern were found in mice which had tumor at site of AK graft and not of Z graft, but some occurred also in other groups. Paradoxically, one with AK pattern had a tumor at the Z graft site only. Most tumors appeared to be of AK/Z type, according to the transplantation pattern. It is possible that many donor-leukemias were of mixed cell type.

Discussion. These experiments confirm the effectiveness of thymectomy in preventing virus-induced leukemia(5,6,7) and the capacity of normal thymic grafts to restore partially the leukemogenic action of the virus (11). The low number of leukemias in grafted Z animals may be due to grafting only one lobe of normal thymus and some resistance of Z as compared to AK/Z mice. In most grafted non-leukemic mice, the grafts could not be identified at autopsy. In the second experiment using AK/Z mice the leukemia incidence in the thymectomized group receiving grafts was over 3 times that in the thymectomized littermates not given grafts (55% and 16% respectively). In the first experiment the sex of donor and recipients was not matched. Female mice may reject male grafts(12). In the second experiment most mice received matching grafts and many received 2 grafts in order to insure a match. However, the importance of matching the sex of the grafted thymus with that of recipient is not indicated by these figures. Leukemia occurred in greater frequency (86%) among the 14 females that received a second grafting (4 of 8 females grafted once *vs.* 12 of 14 grafted twice) but females are known to be more susceptible to thymic lymphoma than males(8). The additional amount of grafted thymic tissue may have been more important than the sex of the donor.

The mechanism by which thymic grafts in virus-injected thymectomized mice restore

the leukemogenic action of the virus is still unknown. A similar problem occurs with x-ray induced leukemias, where thymic grafts can restore the capacity for development of leukemia prevented in thymectomized irradiated mice. In Kaplan's work, all leukemias bioassayed appeared to be of the graft origin (13); in studies of Law and Potter(14), 64% appeared to originate in the hybrid host tissue under the influence of some factor supplied by grafted thymus. This is comparable to our findings, suggesting that a thymic factor can affect the virus-injected hybrid host and allow the leukemic transformation to occur in the host's tissue. This factor may be related to the lymphocyte stimulating factor (LSF) of Metcalf(15) which he considers to be a thymic hormone. Attraction exerted by normal thymus grafts and their repopulation by normal as well as malignant lymphocytes of the host have been shown by several workers(14,16,17), as well as in the present studies. Observations that more tumors developed at site of the AK graft than the Z graft, and more of these tumors appeared genetically of AK cell type than of Z type, may be related to the greater genetic susceptibility of AK thymus to malignant transformation, or perhaps to richness in virus of the preleukemic AK thymus.

The results of many transplantation assays here recorded are not clear-cut and further work is needed to explain the puzzling findings. An unknown number of virus-induced leukemias may have been of mixed cell type. This supposition is supported by the observation that on subpassage some of the tumors failed to maintain the initial histocompatibility pattern. However, neoplastic transformation may yield cells with immunological patterns different from that of normal (non-leukemic) cells.

Summary. 1. Passage leukemia virus A of Gross produced leukemia in 15 of 22 virus-injected intact Z mice, 0 of 17 virus-injected thymectomized, and 2 of 20 virus-injected thymectomized Z mice given one lobe of normal thymus from uninjected donors of the same strain. 2. Thymuses of virus-injected mice removed at thymectomy, when implanted in 19 uninjected thymectomized adult Z mice, produced 2 lymphomas in the grafts. 3. Virus was found by bioassay to persist in tissues of virus-injected thymectomized non-leukemic Z mice sacrificed at 8 to 9 months of age. 4. In similar experiments in AK/Z mice, leukemia occurred in 14 of 15 virus-injected intact mice, 4 of 25 virus-injected thymectomized, and 24 of 44 virus-injected thymectomized AK/Z mice given normal thymus grafts from young AK and Z donors. 5. Bioassays were performed for immunogenetic character of 22 leukemias and tumors arising in thymic grafts on virus-injected thymectomized AK/Z mice. AK, Z and AK/Z recipients were used with the following results: a) 3 leukemias failed to grow in any recipients. b) In 8 cases, grafts took in AK/Z only (excluding those with uncertain typing); in 4 cases, in AK and AK/Z mice; and in one case in Z and AK/Z recipients. 6. These data indicate that a thymic factor, other than cell, supplied by normal thymus grafts may carry to completion viral leukemogenesis inhibited by removal of the thymus.

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Effect of Oxalate on Turnover of Radioactive Phosphate by the Human Erythrocyte.*†‡ (26360)

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Although oxalate has been reported to be involved in glycolysis of human blood(1,2,3, 4,5,6,7), its specific role has not been completely determined. The present investigation was made to study further the effects of oxalate on uptake, distribution and loss of phosphate by the human erythrocyte using radioactive phosphate and radioactive oxalate. Distribution of oxalate between plasma and erythrocyte, turnover of phosphate by erythrocytes incubated with oxalate, role of potassium, magnesium, and calcium ions on the effect of oxalate upon phosphate metabolism, and the effect of oxalate on distribution of phosphate in some of the acid-soluble phosphate ester fractions of the erythrocyte were determined.

Method and materials. Blood drawn from young adults was stored in sterile bottles containing sodium heparinate. The pH of the fresh blood was measured with a Beckman Model G pH meter with a glass electrode. During incubation the blood was kept mixed and at a nearly constant pH of 7.4 by bubbling a gas mixture containing 95% oxygen and 5% carbon dioxide slowly in through a finebore glass tube.

Since Weinhouse and Friedmann(12) demonstrated that oxalate is not metabolized in whole blood, radioactive oxalate (0.00075 Molar) was added and its distribution in whole blood determined by measuring the radioactivity of plasma, hemolyzed whole blood, and hemolyzed washed red cells. Radioactive analysis of a paper electrophoretic chromatogram from the hemolyzed whole blood was done to establish whether or not the oxalate was bound to protein.

Radioactive phosphate was used to measure turnover of phosphate by erythrocytes as affected by oxalate alone or in conjunction with calcium, magnesium, and potassium ions. Fresh erythrocytes were washed with physiological saline phosphate-buffer solution of pH 7.4, incubated for 30 minutes at 37.5°C in their own serum or the above mentioned physiological solution containing radioactive P^{32} inorganic phosphate, washed, then 1 volume further incubated one hour at 37.5°C in 50 volumes of saline-glycine buffer (0.15 molar NaCl, 0.0024 molar glycine, pH = 7.4). After the second incubation the supernatant solution was withdrawn and erythrocytes were washed 3 times with saline, then hemolyzed with 0.01 Molar sodium carbonate in 27% ethanol. P^{32} activity per volume of supernatant and cell hemolysate was determined. These data were used in the equation below for determining the fraction loss. In view of the constancy of turnover rate at one hour, this single determination was used for the assay. Since loss-rates varied with concentration of the phosphate in the uptake media, a standard physiological saline phosphate-buffer medium [0.30% glucose, 0.09% NaCl, 0.15 molar NaH_2PO_4-Na_2HPO_4, pH 7.4], which was found to give reproducible turnover rates, was used throughout the experiment. All tested uptake media were compared to it. Radioactivity measurements were made on "infinitely thick liquid specimens" using a lead shielded

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‡ Radioactive phosphate used in this study was furnished on allocation by the Atomic Energy Comm. at 20% of cost.

thin-walled glass Geiger-Müller counter. Counting time sufficient to give a standard deviation of less than 3% was used.

Whole blood was incubated with or without added oxalate (0.00075 molar) over a period of 4 hours. Uptake of radioactive phosphate by the acid-soluble phosphate esters of the erythrocytes was determined after separation by chromatography(9). Total phosphate of the esters was determined on the wet washed samples(10) by the method of Berenblum and Chain(11).

TABLE I. Distribution of C^{14} Oxalate after 4 Hours Incubation at 37.5°C with Whole Blood.

Blood fraction	Counts/min./ml
Plasma	205 ± 6
Washed cells	415 ± 9
Initial counts for whole blood	288 ± 6
$\left(\frac{\text{cells}}{\text{plasma}} = 42\%\right)$	

Values indicated (±) are stand. dev. of means of 4 samples.

Results of the present studies are shown in Tables I, II, III, and IV and Fig. 1. Oxalate was distributed significantly in favor of the erythrocytes (Table I).

In Table II, per cent of radioactivity lost from erythrocytes previously incubated with radioactive phosphate is expressed by the equation:

$$\text{Fraction lost} = \frac{\text{Activity of supernatant sol.}}{\text{Activity of supernatant sol.} + \text{activity of erythrocyte hemolysate}}.$$

Rate of P^{32} lost from red cell is dependent only on the P^{32} present in the red cell.

$$\left(-\frac{dA}{dt} = kA;\ A = \text{remaining activity}\right)$$

Varying the concentration of oxalate in the incubation medium markedly affects loss of radioactive phosphate from the washed erythrocytes (Fig. 1). The results show that above a concentration of 0.00075 molar oxalate the loss rate becomes constant. There is no significant change in rate of radioactive phosphate uptake among the uptake incubation media containing different concentrations of oxalate.

TABLE II. Rate of Loss of P^{32} from Erythrocytes after Incubation with P^{32} Phosphate.

Time (hr)	% P^{32} remaining in cells after incubation in: Serum	Saline-phosphate buffer	Specific reaction rate constant [k = (conc./time) / (avg conc.) = hr^{-1}]: Serum	Saline-phosphate buffer
0	100	100		
.50	82	76	.397	.546
1.00	67	58	.400	.544
1.50	55	45	.399	.533

Values indicated are avg of 4 trials.

Potassium, magnesium, and calcium ions affect oxalate inhibition of phosphate loss (Table III).

The effect of oxalate on distribution of phosphate into the adenosine-triphosphate (ATP), adenosinediphosphate (ADP), combined hexose-triose phosphates (H – 6 – P + G – 3 – P), 2, 3-diphosphoglyceric acid(2,3-DPGA), and inorganic phosphate (Pi) fractions of the acid-soluble phosphate ester fraction of the erythrocyte was studied. Table IV shows distribution of phosphate and radioactive phosphate in these fractions. These results have interpretive value in view of the demonstration by Gourley(13) of a lack of non-metabolic phosphate exchange between inorganic phosphate and glycolytic intermediates. Other oxalate effects that have been described in erythrocytes, such as decreased glucose utilization(1) and decreased enolase activity(4), have been verified in this laboratory. In this investigation ADP and ATP concentrations, their P^{32} ac-

TABLE III. Relative Loss of P^{32} from Washed Human Erythrocytes after (1) Incubation in Physiological Phosphate-Saline Buffer (Control) and (2) Incubation in the Buffer Plus Added Cations.

Molarity of added ions in incubation medium: K	Mg	Ca	Relative loss: Control	Oxalate
			100	62
.006			100	56
	.0015		100	94
		.005	136	136
.006	.0015		100	62
		.005	136	117
	.0015	.005	100	100
.006	.0015	.005	103	100

Values are avg of 4 trials.

TABLE IV. Effect of Oxalate on Abundance of Erythrocyte Phosphates after 4 Hours Incubation of Human Blood at 37.5°C with $P^{32}O_4$.

Fraction	Control			With oxalate		
	P^{32}	P^{31}	P^{32}/P^{31}	P^{32}	P^{31}	P^{32}/P^{31}
ADP	125 ±11.0	46 ± 2.8	2.70 ± .32	86 ± 4.7	54 ± 3.7	1.60 ± .05
ATP	114 ± 1.8	48 ± .4	2.40 ± .04	76 ± 7.0	46 ± 2.8	1.70 ± .05
H-6-P – G-3-P	54 ± 4.2	24 ± 1.6	2.30 ± .25	92 ± 5.3	37 ± 3.7	2.50 ± .32
2,3-DPGA	472 ± 6.1	202 ±21.0	2.30 ± .20	612 ± 9.0	245 ± 3.7	2.40 ± .06
$P_{inorg.}$	64 ± 5.8	39 ± .4	1.60 ± .12	39 ± 2.2	22 ± 1.6	1.80 ± .19

P^{32} values in counts/sec./ml blood cells (vol of blood cells detn. from original hematocrit).
P^{31} values in μg/ml blood cells.
Values indicated (±) are stand. dev. of means of 4 samples.

tivities, and their specific activities were lowered: inorganic phosphate concentration and its P^{32} activity were lowered while its specific activity was slightly raised; the 2,3-DPGA concentration, its P^{32} activity, and its specific activity were all raised; and concentration of the combined (H – 6 – P and G – 3 – P) fraction, its P^{32} activity, and its specific activity were all raised.

Discussion. It was demonstrated that oxalate concentrates in the erythrocyte, and by paper electrophoresis that none of the oxalate of the plasma, hemolyzed washed cells, or hemolyzed whole blood migrated with the albumins, globulins, or hemoglobin. It is postulated that oxalate occurs in the erythrocyte in an unbound form capable of reacting with glycolytic cofactors to bring about a lowering of phosphate loss from the erythrocytes (Table II).

In general, magnesium or calcium, alone or in combination, removed the oxalate effect and potassium enhanced it slightly. Since the glycolytic metabolism of inorganic phosphate is considered to be magnesium-dependent, it might be postulated that oxalate produces its effect by tying up magnesium. Addition of excess magnesium or calcium overcomes all of the oxalate effect.

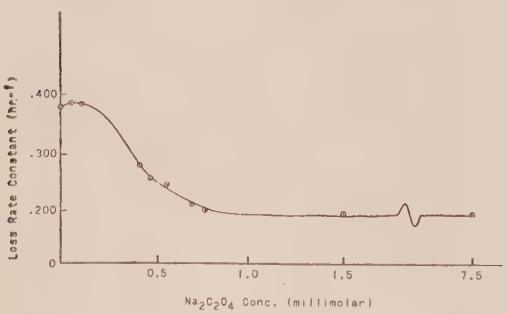

FIG. 1. P^{32} loss rates after incubation with P^{32} phosphate and varying amounts of sodium oxalate. Values on graph are avg of 4 samples with a maximum stand. dev. from means of ± 5%.

The effect of oxalate on distribution of phosphate in the acid-soluble phosphate ester fractions of the erythrocyte may in each case be attributed directly or indirectly to those reactions in the "Embden-Meyerhof" glycolysis scheme that depend on available magnesium. This is further evidence to support the theory that oxalate binds magnesium thus rendering it unreactive as a co-factor.

Summary. When incubated with human blood, C-14 oxalate is distributed between cells and plasma with a significant preference for the cells. After incubation with inorganic P^{32} phosphate and oxalate, rate of loss of P^{32} from the erythrocytes suspended in buffered-normal saline was lower in the case of erythrocytes pre-incubated with oxalate, reaching a minimum value with 0.00075 molar oxalate. Inhibition of P^{32} loss from erythrocytes by oxalate was slightly enhanced by potassium but was reversed by calcium and magnesium ions in physiological concentrations. Concentration of the cellular organic phosphates of the acid-soluble ester fraction changes significantly after incubation of erythrocytes with inorganic P^{32} and oxalate. The changes caused by oxalate may in each case be attributed directly or indirectly to a reduction in available magnesium.

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Constant Relation Between Blood Level of Thyrotropin and Total Thyrotropin in Thyroidectomized Mice. (26361)

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Several reviews concerning regulation of thyrotropin (TSH) release have recently appeared(1-4). A dual control seems to exist. One is a fundamental, non-neural regulatory mechanism, while the other is a neural mechanism for emergency release of TSH.

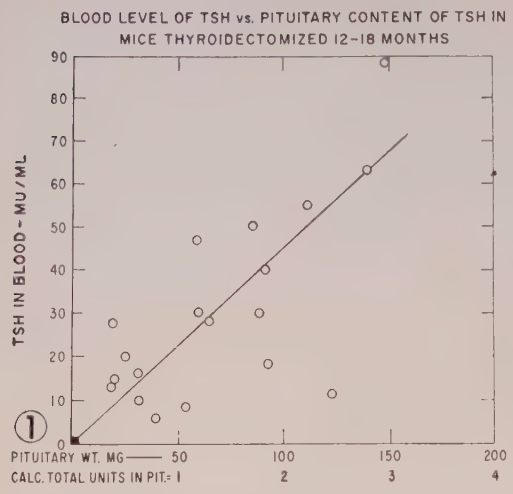
This paper presents quantitative data concerning the non-neural release of TSH. Levels of TSH in blood and pituitary or tumor were determined in two kinds of radiothyroidectomized mice, (1) those with enlarged pituitaries only, and (2) those with transplantable, TSH-producing tumors of pituitary origin. In both cases the TSH level in the blood was proportional to total amount of TSH in the pituitary or the tumor.

Methods. All mice were of the C57Bl strain and were radiothyroidectomized at about 3 months of age using approximately 200 μc of I^{131}. The 2 transplantable pituitary tumor lines 77D and 101D were obtained originally from Dr. Jacob Furth(5) and were carried for 11 and 7 transfer generations, respectively. For each transfer, suspensions of tumor tissue were injected subcutaneously into the hind leg of mice, radiothyroidectomized about one month previously. Tumors weighing 2 to 8 g developed after 6 or more months.

TSH was measured in baby chicks by thyroidal I^{131} depletion(6). Potencies are in terms of USP units. All pituitary and tumor tissues were frozen as soon as possible after removal and then lyophilized and milled to a 60 mesh powder in a Wiley mill. A suspension of this powder in 0.1 M $NaHCO_3$ was injected into chicks for estimation of potency. Heparin was added to all blood samples. Samples of blood ($>$ 1 ml) obtained from the vena cava of mice with tumors were pooled, centrifuged and the plasma used for bioassay. Blood samples, ($<$ 1 ml) obtained from individual mice with enlarged pituitaries, were assayed directly.

Results. Blood level of TSH in thyroidectomized mice with large pituitaries. The pituitaries of mice become greatly enlarged 12-18 months after radiothyroidectomy(7). Nineteen such mice with large pituitaries were killed. The blood was collected and assayed. The pituitaries were weighed and either assayed or transplanted to start new tumor lines. The positive relationship between pituitary weight and concentration of TSH in the blood is shown in Fig. 1. Most of the pituitaries that were assayed had a potency between 0.1 and 0.2 U of TSH per mg of dry pituitary weight. Hence, a unitage scale has been added to facilitate comparison with Fig. 2. The small square at the origin represents values estimated for TSH levels in normal mice (about 1 mU/ml of blood and 40 mU/2 mg of pituitary).

The milliunits of TSH per ml of blood



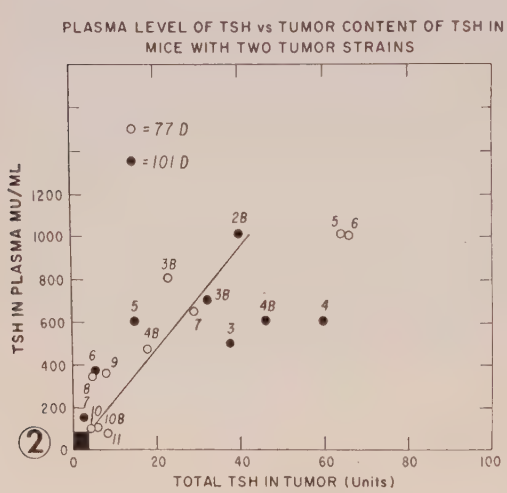


FIG. 1. Relationship of blood level of TSH to pituitary size and TSH content in mice thyroidectomized 12-18 mo. The line has a slope of 25 mU/ml/unit of TSH in the pituitary. Each point represents data from one mouse. Square at origin represents data from normal mice.

FIG. 2. Relationship of plasma level of TSH to TSH content of two strains or lines of tumors, 77D and 101D. Square at origin represents area covered by data in Fig. 1. Each point represents data obtained on pooled plasma and pooled tumor tissue from many mice. Numbers refer to transfer generation of the tumor. The line has a slope of 25 mU/ml/unit in tumor.

was divided by the calculated TSH content of the pituitary in units for each of the 19 mice. The average of these potency ratios was 25.6 ± 4 mU/ml of blood/unit of TSH in the pituitary. A line with this slope of 25 mU/ml/unit in the pituitary has been drawn in Fig. 1 to represent the average relationship found.

Plasma level of TSH in mice with TSH-producing tumors. In Fig. 2 the milliunits of TSH/ml of plasma from tumor-bearing mice are plotted against total amount of TSH in tumors of average size for each of several generations. Each point represents data from assays of a pool of plasma and a pool of tumors from many mice. The potency of the tumors decreased markedly in the later generations. As the potency of the tumors decreased, so did concentration of TSH in the blood. The small rectangle at the origin covers the area of this graph which would include the data presented in Fig. 1. A line has been drawn which has the same apparent slope as the one in Fig. 1. A curved line might fit the points better, but there is considerable scatter. The mean potency ratio for the 19 different tumor generations was 33 ± 3.5 milliunits per ml of plasma per unit of TSH in the tumor.

The above data were from old tumor lines that had already lost much potency by the time tests were begun. New tumor lines are now being established in our laboratory. These vary greatly in potency. Tumors of the first transfer of our tumor line W were especially potent. Three mice with this tumor had 5 g tumors having a potency of 0.23 U/mg dry weight or 230 U/tumor. Plasma level of TSH was 14 U/ml. The potency ratio $\left(\frac{14{,}000}{230}\right)$ obtained in these mice was 61 mU/ml of plasma/unit in the tumor, or about 30 mU/ml of blood/unit in the tumor. This is very similar to the ratio of 25 found in the mice without tumors. If plotted, this point (230 U/tumor × 14 U/ml blood) would require a graph 10 times the size of Fig. 2, or to another order of magnitude larger. The potency of lyophilized plasma from these animals was 0.14 U/mg, a most remarkable potency because it is equal to TSH concentration in the pituitary of the normal mouse and of most tumor tissues per milligram dry weight.

Inhibition of TSH production by thyroxine. Several mice with large TSH tumors were injected with 10 μg of L-thyroxine a day for 4 days. In all cases the blood level of

TSH dropped to 10-30% of the starting level. One mouse with a 1 g tumor survived daily injections of 10 μg of thyroxine for 72 days, when it was killed. During this time the tumor did not change in size. Plasma TSH level at death was still 40 mU/ml. This was 4% of the TSH level in the same mouse before thyroxine injection, but was 40 times the blood level in normal mice. Also, the concentration of TSH in the tumor was found to be 4% of that in other tumors from mice of the same generation that were not treated with thyroxine. The potency ratio after thyroxine for 72 days was 30 mU/ml of blood/unit in the tumor.

Effect of TSH level on hypertrophy of the pituitary. The high blood level of TSH in mice with tumors does not prevent the hypertrophy of the pituitary that begins about a year after thyroidectomy. Tumors of strain 101-D, generation 4, grew so slowly that the tumors of 19 mice were not harvested until 11 months after thyroidectomy, about 10 months after transfer. Average pituitary weight of these 19 mice was 9.8 mg, which is more than 5 times the normal weight of the pituitary. TSH concentration in the pituitaries was 0.1 U/mg dry weight, which is essentially normal. These data clearly show that the blood level of TSH does not inhibit the factor or factors causing pituitary hypertrophy in thyroidectomized mice. The physiological inhibiting agent is known to be thyroid hormone(8,9), produced by the thyroid and obviously absent in these thyroidectomized mice.

Discussion. It is of interest that the relation between blood level of TSH and total amount of TSH in the body of these thyroidectomized mice varied on the average by a factor of not more than 2, whether the mice were treated with thyroxine or not and whether the primary source of the TSH was the pituitary *in situ* or a tumor in the leg, even though the level of TSH in the plasma ranged from 1 to 14,000 mU/ml. Thus in thyroidectomized mice the blood level of TSH reflects almost quantitatively the total stores of TSH in the body, whether in the pituitary or in a tumor in the leg. This relation, of course, may be altered temporarily in a normal mouse by hypothalamic stimulation. But in the case of the mice with tumors in the leg the release of TSH is surely independent of hypothalamic control.

No appreciable storage of TSH in body tissues outside of the pituitary gland and pituitary tumors has been found(10). The blood level of approximately 25 mU/ml/unit of TSH in the pituitary or tumor, when viewed conversely, indicates that the blood level of TSH in U/ml multiplied by 40 gives an estimate of total units of TSH stored in the pituitary or tumor tissue of these mice. Since the blood volume of a mouse is less than 3 ml, it follows that 90-95% of total TSH in the body is estimated to be in the pituitary or tumor tissue.

The pituitary is still of normal size (<2 mg) in most mice with tumors, so that the 20 to 40 milliunits present in the pituitary contribute very little to the total amount of TSH in the body. In a mouse with a tumor the total amount of TSH in the blood (about 1 unit) is usually less than 5% of that in the tumor, but some 50 times that in the pituitary.

Kumaoka, Money, and Rawson(11) have recently studied the inhibitory effect of thyroxine and thyroxine analogues upon growth of TSH-producing tumors similar to those used here. Combining their studies with those reported here, it can be stated that in mice with TSH-producing tumors, thyroxine injections at a high dosage level(1) inhibit or stop tumor growth; (2) lower TSH levels in blood to about 5% of the initial level, but not to normal levels, and (3) lower TSH concentration in the tumor to about 5% of initial concentration, but not to zero.

Summary. In thyroidectomized mice approximately the same relationship exists between the blood level of TSH and total amount of TSH in the TSH-producing pituitary tissue in the body whether this tissue is *in situ* in the sella turcica or in a tumor in the hind leg, and while the amount of TSH in this tissue varies by 10,000 times from an amount of 20 milliunits to 200 units. These results demonstrate clearly and quantitatively the autonomous release of pituitary TSH independent of any hypothalamic in-

fluence. In these mice the units of TSH/ml of blood times 40 gives an approximate measure of total TSH in units in the body. Thyroxine treatment caused a parallel lowering of the level of TSH in blood and tumor.

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Identification of Extrahepatic Bilirubin Monoglucuronide and Its Conversion to Pigment 2 by Isolated Liver. (26362)

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Bollman and Mann(1) noted that as the amount of serum bilirubin increased after hepatectomy in dogs, the van den Bergh reaction changed from indirect to direct, and bile pigment began to appear in the urine. This indicated that the liver was not the only organ capable of forming bilirubin or converting it to direct bilirubin. Later, work by Billing and Lathe(2), Schmid(3), Talafant(4) and Schachter(5) indicated that conjugation of bilirubin prior to its excretion involves solubilization and accounts for the nature of the reaction of the pigments in the van den Bergh test. The indirect reaction occurs with the water-insoluble free bilirubin, whereas the direct reaction comprises pigment 1 and pigment 2, the water soluble conjugates of bilirubin. Pigments 1 and 2, as obtained by reversed phase partition chromatography, have been described as bilirubin monoglucuronide and diglucuronide, respectively. However, other nonglucuronide conjugates of bilirubin also may be found in pigment 1 and 2 fractions. Bollman(6) observed that the direct pigment in the plasma of the hepatectomized dog has the characteristics of pigment 1 with respect to chromatographic mobility and diazotization reaction. Whether this pigment is a glucuronide or some other conjugate of bilirubin had not been established(7). The present report determines this conjugated pigment of extrahepatic origin to be bilirubin monoglucuronide.

Methods and procedures. Total hepatectomy was performed as previously described by Grindlay and Mann(8), the dog being maintained postoperatively by a saline-glucose solution administered by a constant injection apparatus. Blood was collected for analysis of the plasma pigments at 42 hours after complete removal of the liver.

The proteins were precipitated from the plasma using ethyl alcohol for 30 minutes. After centrifugation, the supernatant was evaporated *in vacuo* and aliquots were dissolved in 0.5 ml of the mobile phase of the solvent system used in the chromatographic separation. It was noted that, as found by Hoffman and associates(9), when ammonium sulfate was used in addition to ethanol for precipitation of the proteins from the plasma of the liverless dog, the pigment appeared to remain bound to the ammonium sulfate crys-

TABLE I. Reactions of Pigment 1 (Isolated by Columns).

Procedure	Glucuronide determination	Direct bilirubin determination			
		NaOH then diazotized	H_2O then diazotized	Diazotized then NaOH	Diazotized then H_2O
mg/100 ml	.48	0	1.3	.564	1.2
10^{-7} moles	247	—	219	—	253
Molar ratio of glucuronide to pigment	—	—	1.13	—	.98

tals and it was not possible to redissolve the pigment sufficiently for satisfactory chromatography. With use of ethyl alcohol only, there was no difficulty in bringing the pigment to solution.

Siliconized kieselguhr was prepared according to the method of Howard and Martin(10). Reversed-phase partition chromatography of the plasma pigments was performed by the method used by Cole and associates(11) and the solvent system used was the butanol-phosphate buffer pH 6 system for separation of the 3 bile pigments.

Three simultaneous columns, with 1 ml of plasma from the hepatectomized dog for each determination, were studied under the following conditions:

The pigment band was washed from the kieselguhr with 5.5 ml of distilled water and separated by filtration. Five milliliters of this substance was utilized for quantitation of glucuronides by the naphthoresorcinol reaction by the method of Fishman and Green (12). Determinations were done in duplicate.

The pigment band was washed from the kieselguhr with 4.5 ml of distilled water and separated by filtration. Two equal aliquots (2 ml each) were added to an equal amount of tenth-normal sodium hydroxide and distilled water respectively. After 30 minutes, diazotized sulfanilic acid was added to both in amounts for determination of the "30-minute direct" van den Bergh reaction, correcting calculations to conform to the modification of Malloy and Evelyn(13).

The pigment band was washed from the kieselguhr with 5 ml of diazotized sulfanilic acid and separated by filtration. Two equal aliquots (2 ml each) were added to an equal amount of tenth-normal sodium hydroxide and distilled water respectively for 30 minutes. The diazotized pigment in each was then estimated with the Beckman spectrophotometer.

A total of 35 ml of plasma from the 42-hour hepatectomized dog was chromatographed on a large kieselguhr column and the single mobile band of pigment was eluted from the column, with the mobile phase of the butanol phosphate buffer pH 6 solvent system. This pigment was dried *in vacuo* at 30°C and reconstituted with rat plasma. The material was then injected into the perfused isolated rat liver system after a control study had been done on the bile pigments. This apparatus was modeled after that described by Brauer and co-workers(14). The bile and plasma were analyzed for the change in amount and type of pigment at 4 hourly intervals after injection of the pigment.

Results. The bilirubin of the hepatectomized dog was 8.5 mg total and 5.0 mg direct for each 100 ml of plasma. The results of the studies of the 3 simultaneous columns prepared from the plasma of the hepatectomized dog are shown in Table I. When the pigment was first treated with sodium hydroxide and then diazotized, all of the pigment was found to be labile to the alkali treatment. However, when the pigment was first diazotized and then treated with alkali, about half (47%) was found to be alkali stable. The molar ratio of glucuronide to pigment (expressed as bilirubin) was approximately 1. Internal controls were inherent in the system.

Table II illustrates the increase in bile pigment and specifically of pigment 2 as measured in the bile collected *via* the cannulated bile duct. This increment occurred after the pigment 1 obtained from the plasma

TABLE II. Biliary Excretion (Perfused Liver) after Pigment 1.

Hourly interval	Bile vol, ml	Direct pigment in bile, mg/100 ml	Pigment 2 in bile, mg/100 ml
Preinj. control	.7	7.6	4.2
1st	.7	15.9	6.1
2nd	.52	19.4	6.9
3rd	.5	19.0	5.5
4th	.5	7.3	3.9

of the hepatectomized dog had been injected into the perfused preparation. Control values were reached again 4 hours after the injection. In control experiments without injection of pigment 1 into the perfusion apparatus, excretion of pigment during a similar 4-hour period remained essentially the same as during the first hour of experiment. After losses of pigment during isolation of pigment 1, it was estimated that 0.18 mg of pigment 1 was injected into the perfusion system. All pigment was removed from the perfused blood and no pigment 1 was found in the bile. Over a 3-hour period 95% of the injected pigment 1 was recovered as pigment 2 in the bile as determined by the increment (0.17 mg) in the direct van den Bergh reaction and chromatography of the bile. The figures given for pigment 2 recovered by column chromatography indicate the loss of this pigment during the procedure. Were any appreciable amounts of pigment 1 present, however, they would have been detected by the columns.

Comment. Both the 42-hour survival and the high levels of plasma bilirubin of the hepatectomized animal afforded the opportunity to carry out these studies. This level of total plasma bilirubin is equivalent to that found after obstruction of the common bile duct for a period of similar duration.

Isselbacher and McCarthy(15) showed that about 15% of the conjugated azopigment in bile is an alkali-stable sulfate conjugate. Work in progress in this laboratory indicates that the sulfate conjugate migrates with the pigment 2 band, whereas no alkali-stable sulfate component is found in pigment 1. Pigment 1 can be accounted for by the monoglucuronide structure. Thus, in this study, the lability of the pigment from the plasma of the hepatectomized dog is compatible with a monoglucuronide structure.

Overbeek and associates(16), in studying the kinetics of the van den Bergh reaction, concluded that after bilirubin is split at the methene bridge, both of the dipyrrol compounds couple with the diazonium salt. Thus, when the pigment from the plasma of the hepatectomized dog is diazotized prior to alkali treatment, only that half of the molecule esterified with the glucuronic acid is labile to the sodium hydroxide. In this situation, alkali lability of 50% speaks for the monoglucuronide structure.

The indications of a monoglucuronide structure that have been mentioned were confirmed when a molecular ratio of 1:1 for glucuronic acid to bilirubin was found in the pigment.

Summary and conclusions. The pigment obtained by reversed-phase partition chromatography from the plasma of the hepatectomized dog has been shown to be bilirubin monoglucuronide (pigment 1). In the experiments performed, pigment 1 was of extrahepatic origin. The isolated perfused rat liver converts bilirubin monoglucuronide to bilirubin diglucuronide and excretes the diconjugate in the bile.

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Parabiotic Anemia-Polycythemia.* (26363)

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Surgical parabiosis of genetically diverse animals frequently results in severe and persistent anemia of one partner and polycythemia of the other(1,2,3). This may be observed as early as the third day after junction. In the absence of genetic diversity this is rare, and, if present, only mild and of short duration. Anemia-polycythemia (AP) has been most commonly observed in instances of unilateral genetic diversity, *i.e.*, after parabiosis of a mouse of one inbred strain with its F_1 hybrid(4). AP has been interpreted generally as due to an immune response of the inbred mouse against the unrelated parent component of the hybrid. In this combination the hybrid becomes anemic and the inbred mouse polycythemic, a development probably brought about by a shift of red cell mass from the hybrid into the inbred mouse. If the AP were the result of an immune response to incompatible tissue, it could be used as an assay of this response. However, this assay should only be of aid in unilateral immune responses. Bilateral immune responses, both aiming at AP, should tend to cancel each other.

The data presented here were obtained from mice of 2 unrelated strains, parabiosed to teach other and to their common F_1 hybrids. They suggest that at least 2 separate factors operate to bring about AP, only one of them being immunologic in nature.

Material and Methods. Experimental animals were inbred mice from our colonies, of the C57BL/6 and BALB/c strains, and their reciprocal hybrids. Partners were of the same sex, with approximately equal numbers of male and female pairs. The technique of parabiosis has been described(3). Blood for microhematocrit determination was obtained from blood of the tail vein. The mice were bled on the 6th, 10th, 13th, and 17th days after parabiosis.

Observations. Parabiosis of *BALB/c mice with* $(C57BL/6 \times BALB/c)F_1$ hybrids resulted in AP on the 6th day in all pairs, invariably "favoring" (= creating polycythemia in) the BALB/c partner (Table 1). It increased in severity until the end of the 3rd week when death had occurred in most instances.

Parabiosis of *C57BL/6 mice with* $(C57BL/6 \times BALB/c)F_1$ hybrids did not result in similar AP on the 6th day. Instead, 18 of 25 pairs showed a moderate AP favoring the *hybrid* partner (mean: 15.7 S.D. $\pm$ 8.4), while the remaining 7 pairs showed mild AP favoring the C57BL/6 (mean: 9.4, S.D. $\pm$ 8.1). However, the trend reversed itself on successive bleeding days; results became uniform, all pairs showing AP favoring the C57BL/6 side, but significantly less severe than in the BALB/c-hybrid combination ($p<0.02$).

Parabiosis of *BALB/c with C57BL/6* mice

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TABLE I. Hematocrit Values of Parabiosed Incompatible Mice.

Day	No. of pairs	Mean hematocrit		Mean hematocrit difference
		BALB/c	F_1	
	(a) BALB/c – (BALB/c × C57BL/6)F_1			
6	13	65.0 ± 3.26*	38.8 ± 5.36*	26.2 ± 6.75*
10	17	74.8 ± 4.79	21.3 ± 6.94	53.5 ± 34.0
13	7	75.6 ± 5.11	18.7 ± 4.46	56.9 ± 6.41
17	9	73.9 ± 18.10	20.6 ± 17.10	53.3 ± 36.90
	(b) C57BL/6 – (BALB/c × C57BL/6)F_1			
		C57BL/6	F_1	
6	25	45.4 ± 8.21*	54.0 ± 7.38*	−8.6† ± 14.09*
10	16	57.9 ± 14.80	29.9 ± 10.4	28.0 ± 22.30
13	14	58.7 ± 11.90	22.3 ± 8.62	36.4 ± 19.27
17	11	55.7 ± 11.3	23.0 ± 11.30	32.7 ± 22.05

Day	No. of pairs	Mean hematocrit		Mean hematocrit difference
		BALB/c	C57BL/6	
	(c) BALB/c – C57BL/6			
6	15	64.7 ± 3.32*	29.0 ± 9.78*	35.7 ± 12.80*
10	7	66.0 ± 10.40	29.7 ± 12.0	36.3 ± 23.84
13	6	61.7 ± 11.40	33.2 ± 11.0	28.5 ± 22.50
17	5	55.2 ± 9.69	37.0 ± 5.02	18.2 ± 15.50

(d) Normal hematocrit values of strains

	No. of mice	
BALB/c	51	50.3 ± 2.89*
C57BL/6	8	51.8 ± 1.66
(BALB/c × C57BL/6)F_1	42	51.0 ± 1.70

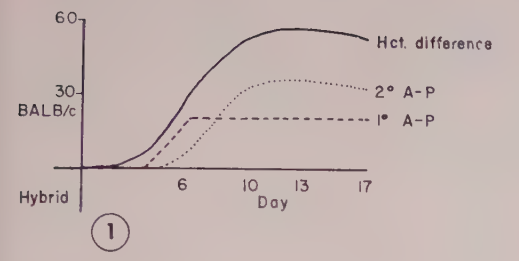
* Stand. dev.

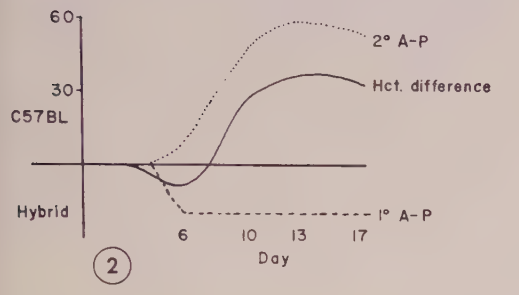
† Hybrid polycythemia.

resulted in AP on the 6th day in all pairs, which uniformly favored the BALB/c partner, and was significantly more severe than in the BALB/c-hybrid combination at that time ($p<0.02$). However, it failed to increase in severity, and on the 10th day was significantly below that seen in the BALB/c-hybrid combination ($p<0.02$). AP persisted at about the same level until the 13th day, after which it decreased somewhat.

Discussion. The results indicate that factors other than, or in addition to, mutual immune responses (BALB/c vs. C57BL/6, and C57BL/6 vs. BALB/c) operate to bring about AP in parabiosis. 1) The BALB/c-C57BL/6 combination brought about more severe AP on the 6th day than either pure-hybrid combination. Mutual immune responses, competing with each other, would have had the opposite effect. The initially lesser degree, when one of the partners was a hybrid, suggests a genetic dilution effect that is usually not associated with genetically determined antigens concerned with tissue incompatibility. 2) The initial AP in the C57BL/6-hybrid combination favoring the hybrid, and followed by its reversal, is not readily explained by an immune response. Polycythemia has been observed in animals *exerting* an immune response against their partners, and hybrids do not react immunologically against their parent strains(3).

We wish to propose the following hypothesis: A *primary* AP may occur between incompatible parabiotic partners as soon as adequate vascular anastomoses have developed. It is unrelated to immune responses, conceivably related to differences in blood pressure between strains, and expresses itself in hybrids in a dilute fashion. It favors the BALB/c over the C57BL/6, their mutual F_1 hybrid occupying an intermediate position. An immune response may give rise to *secondary* AP which is superimposed on the primary, and overrides it. Fig. 1 to 3, in which hematocrit differences are plotted against time, are an attempt to explain the observations diagrammatically on the basis of these postulates. Points at the level of the abscissa indicate absence of a hematocrit difference;





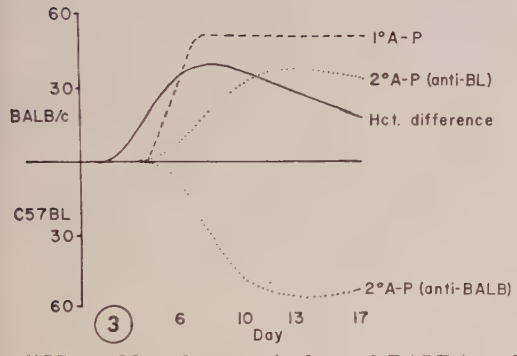


FIG. 1. Mean hematocrit data of BALB/c and (C57BL × BALB/c)F_1 mice joined in parabiosis (see text).

FIG. 2. Mean hematocrit data of C57BL/6 and (C57BL/6 × BALB/c)F_1 mice joined in parabiosis (see text).

FIG. 3. Mean hematocrit data of BALB/c and C57BL/6 mice joined in parabiosis (see text).

points above and below indicate a polycythemia of the partner shown at the left (e.g., in Fig. 1, hematocrit values of BALB/c mice are higher than those of their hybrids).

BALB/c-hybrid (*Fig. 1*): The primary AP (broken line) favors the BALB/c partner, but is of only moderate strength. The secondary AP (dotted line) also favors the BALB/c (since the hybrid can not react against the BALB/c); it begins to make itself felt already on the 6th day, but increases in severity thereafter. It is superimposed on the primary AP as a result of which both anemia and polycythemia are more severe than in the other two combinations.

C57BL/6-hybrid (*Fig. 2*): The primary AP favors the hybrid. The secondary AP favors the C57BL/6, partly cancels the effect of the primary AP on the 6th day, and overrides it on successive bleeding days. It never reaches the level of the AP in the BALB/c-hybrid combination because of the persistent opposing effect of the primary AP.

C57BL/6-BALB/c (*Fig. 3*): The primary AP favors the BALB/c, and is stronger than in the BALB/c-hybrid combination. The secondary responses pull both ways, and essentially cancel each other; in consequence, there is no further increase above the level of the 6th day. The decrease in AP during the 2nd week is probably the result of the readily demonstrable narrowing of vascular anastomoses between bilaterally incompatible partners(4).

Conclusions. Hematocrit data from incompatible mice joined in parabiosis suggest that a primary and a secondary anemia-polycythemia may develop. The secondary anemia-polycythemia appears related to an immune response, while the primary does not. The two events may counterbalance or intensify each other, depending on the genetic backgrounds of the mice. Because of this multiplicity of factors, the anemia-polycythemia of parabiosis appears unsuitable as an assay of the host response to incompatible tissue.

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Colloidal Yttrium Hydroxide-Y-90 and Yttrium Phosphate-Y-90. Preparation and Tissue Distribution Following I. V. Administration. (26364)

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Radiocolloid therapy of leukemia and other malignancies, first reported by Hahn (1) and co-workers, has steadily gained acceptance as an advantageous radiation modality. Of the numerous radioisotope preparations proposed for this purpose, only 3 (Gold-198, chromic phosphate-P-32, and zirconyl phosphate-P-32) have been adequately described and tested(2,3,4). These materials share a common attribute. Following *i.v.* administration they concentrate in reticulo-endothelial tissues where they achieve a selective irradiation while sparing other organs of the body (not possible with external irradiation or with commonly employed ionic radioisotopes). Even these, however, have certain disadvantages. Radiogold is undesirable because of its penetrating gamma emission, while chromic and zirconyl phosphates, though pure beta-emitters, have for certain situations an undesirably long half-life (14.3 days). Thus, the latter produce their biological effects rather slowly and leave appreciable residual activity in the patient for a considerable period. For the treatment of acute and chronic leukemia and in particular for selective sublethal irradiation of leukemic patients prior to attempted bone marrow transplantation, a more rapidly acting radiocolloid which would decay promptly was especially desired. Radioactive yttrium (Y-90) with its short half-life (2.54 days) and its energetic beta emission (2.18 MEV) seems uniquely suited for this purpose.

Simple methods for preparation of stable colloids of yttrium hydroxide and yttrium phosphate have been developed. Their properties, toxicities and tissue deposition following *i.v.* administration have been studied in mice, and terminal leukemic patients. Their therapeutic application is described elsewhere (5).

Methods. Screening tests. Many surfactants and suspending agents were evaluated for their effectiveness in preparation of colloidal yttrium hydroxide and yttrium phosphate *in situ*. Those which permitted flocculation or sedimentation of the colloid within 48 hours were rejected. Of the remainder, carboxymethylcellulose and Igepon T-77† served to disperse both phosphate and hydroxide, while albumin, dextran,‡ maltose, and dextrin were effective only with the latter. Igepon T-77 was eliminated for lack of information as to its toxicity. Albumin was subsequently eliminated when it was found to interfere with complete conversion of yttrium to the insoluble hydroxide form.

Tests of completeness of conversion. Completeness of chemical conversion of radioyttrium to the desired colloidal form was evaluated in low specific activity pilot experiments. By methods such as described below, yttrium hydroxide was prepared in presence of each dispersent listed above, as was yttrium phosphate. After pasteurization at 65°C for one hour, aliquots of each colloid were dialyzed against 250 volumes of physiologic saline at a pH of 8.1. After 24 hours the dialysate and original colloid were assayed for radioactivity, and the percent dialyzable radioyttrium calculated. Results are presented in Table I. From these studies albumin was rejected, and the preparations of yttrium hydroxide in maltose and of phosphate in carboxymethylcellulose were selected for additional study.

Preparation of therapeutic colloidal yttrium hydroxide, $Y(OH)_3$-Y-90. To radio-

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† Antara Chemicals Div., General Dyestuff Corp., New York.

‡ Plavoflex, 6% Aqueous, Wyeth Labs.

active yttrium chloride§ (75-275 millicuries) in its original shipping container, 1.0 ml yttrium chloride carrier solution (0.01 M YCl_3 in 0.1 N · HCl) was added. These were mixed, allowed to equilibrate at room temperature for 10 minutes, and transferred to a suitable 20 ml vial, using sterile hypodermic needle and syringe. The original container was then washed with 5.0 ml of 50% maltose solution, the wash being added to the radioactive material and mixed. A drop of phenolphthalein solution was added, and the mixture titrated to its endpoint with 1.0 N · NaOH. Total volume was adjusted to 10.0 ml with sterile 0.9% NaCl. Sterilization was achieved by pasteurization at 65°C for one hour and the product stored at 5°C.

TABLE I. Dialyzability of Various Radioyttrium Colloids.

Chemical form	Dispersant	% dialyzable Y-90
YPO_4	Carboxymethylcellulose	.02%
$Y(OH)_3$	"	.44
"	Albumin	75.9
"	Dextran	1.8
"	Dextrin	1.2
"	Maltose	1.3

Preparation of therapeutic colloidal Yttrium phosphate, (YPO_4-Y-90). One ml yttrium chloride carrier solution was added to the radioactive yttrium chloride in its original shipping container, mixed, and allowed to equlibrate for 10 minutes at room temperature. The mixture was transferred to a suitable 20 ml vial and the container washed with 5.0 ml 4.0% carboxymethylcellulose solution. The washings were added to the radioactive mix, 0.5 ml of 0.1 M Na_3PO_4 was added, mixed, and the solution titrated to the phenolphthalein endpoint with 1.0 N · NaOH. The volume was adjusted to 10 ml with sterile 0.9% saline, and the product pasteurized at 65°C for one hour and stored at 5°C.

Fate in experimental animals. Tissue distribution of these radiocolloids in mice 1, 3 and 7 days following their *i.v.* administration was evaluated by methods described previously(4).

§ Obtained as carrier-free YCl_3-Y-90 in HCl from Oak Ridge Nat. Labs., Union Carbide Nuclear Co., Oak Ridge, Tenn.

TABLE II. Toxicity of $Y(OH)_3$-Y-90 for Swiss Albino Mice.

Dose (mc/kg)	28-day survival	Dose (mc/kg)	28-day survival
200	.0%	18	.0%
150	.0	16	.0
100	.0	14	.0
50	33.3	12	100.0
40	.0	10	100.0
30	33.3		

Radiation toxicity. Toxicity of yttrium hydroxide-Y-90 was evaluated in white mice by *i.v.* administration of varying doses (10 to 200 mc/kg). These animals were observed during a 28-day period and percent survival in each group calculated, Table II.

Excretion. Total excretion per day of Y-90 by mice was investigated during the study described above. Methods were those described previously(4) (Table III).

Localization in man. Six patients received each radiocolloid. Of these, 11 were terminal acute leukemia, while one had inoperable C.A. of the rectum. Tissue assay methods were those previously described(4). Diagnoses, doses, and survival times of all patients are indicated in Tables IV and V.

Excretion by man. From 9 of the above patients, complete 24 hour urine specimens were collected for assay of excreted Y-90.

Results. These radiocolloids had remarkable stability; after prolonged storage at 5°C, no visible sediment was observed. Electron microscopy revealed well-dispersed, uniform micelles ranging from 0.04 to 0.08 μ in diameter. The products were routinely demonstrated to be sterile by thioglycollate culture methods. In no instances were pyrogenic reactions elicited upon their *i.v.* administration to experimental animals or man. The tissue distribution of maltose-suspended

TABLE III. $Y(OH)_3$ Excretion by Mice.

Day	% excreted
1	.49%
2	.13
3	.05
4	.03
5	.03

TABLE IV. Tissue Deposition of Y-90 in Leukemic Patients after I.V. $Y(OH)_3$.*

Patient	SMB†	MLD†	SR†	VP†	MM	JM
Days survival	17	20	20	23	26	57
Dose, mc	53.3	54.6	75.0	56.9	6.6	20.0
Body wt, kg	25.9	18.1	19.1	15.9	61.0	nm
Diagnosis	L	L	L	L	M	L
Tissue	% of recovered Y-90/g of wet tissue					
Marrow	.0346	.062	.075	.067	.012	.026
Bone	.020	.012	.015	.023	.006	nm
Spleen	.014	.030	.028	.055	.013	.009
Liver	.006	.015	.007	.004	.012	.007
Lung	.0007	.002	nm	.002	.002	.009
Kidney	.004	.020	"	.005	.001	.007
Adrenal	.0001	.002	"	.002	.0006	nm
Heart	.0003	.0005	"	.0002	.0002	.0063
Node	.0005	.004	"	.001	.0008	nm
Muscle	.0001	.0006	"	.0002	.0003	"

* Postmortem results.
† Massive irradiation prior to attempted marrow transplantation.
nm = Not measured. L = Lymphatic. M = Myelogenous.

TABLE V. Tissue Deposition of Y-90 in Patients after I.V. YPO_4.*

Patients	MB	AMH	CDS	LFD	EM†	PM
Days survival	1	3	5	6	21	72
Dose, mc	3.2	2.0	19.2	19.2	125	15
Body wt, kg	61.7	48.5	66.2	63.5	64.9	47.6
Diagnosis	CML	CLL	AML	ALL	CR	AGL
Tissue	% of recovered Y-90/g of wet tissue					
Liver	.021	.012	.010	.006	.006	.005
Marrow	.0052	.0011	.015	.017	.0012	.002
Lung	.005	.014	.0012	.005	.0002	.016
Bone	.001	.001	.0003	.0035	.011	.015
Spleen	nm	.031	.007	.004	.005	.022
Kidney	.0037	.0044	.001	.001	.0007	.0005
Node	.0006	.0005	.0011	.0008	.0002	.0004
Muscle	.0005	.0003	.0007	.0000	nm	nm
Adrenal	.002	.002	.002	.0006	"	"
Heart	.001	.001	.0005	.0002	.0001	"
Brain	.0000	.0000	.0000	nm	.0000	"

* Postmortem results.
† Massive irradiation prior to attempted marrow transplantation.
nm = Not measured. A = Acute. C = Chronic. LL = Lymphatic leukemia.
G = Granulocytic. ML = Myelogenous leukemia. CR = Carcinoma of rectum.

radioyttrium hydroxide in Swiss albino mice is presented in Table VI. The corresponding data for carboxy-methylcellulose-suspended yttrium phosphate in AKR mice are presented in Table VII. Results are reported as percent of recovered activity per gram of wet tissue. Each figure represents the mean of 3 animals for each sacrifice date. Considerable variance among animals of any one group was observed so that small differences between groups probably have little significance. Despite these limitations some conclusions appear warranted. Following *i.v.* administration, both radiocolloids disappeared rapidly from the circulating blood. With both colloids, much of the Y-90 was deposited in liver and in bone (including marrow). Of the remaining tissues, spleen, kidney, adrenal, and lung revealed significant accumulation of radioactivity. Other tissues contained only very minute quantities. These data suggest that both colloids are deposited primarily in the reticulo-endothelial system (presumably by phagocytosis). However the high specific activity of bone is suggestive of a competing mechanism. Efforts at separation of bone and bone marrow for independent assay were unsuccessful, and one is un-

TABLE VI. Tissue Deposition of $Y(OH)_3$ in Mice after I.V. Administration.

Tissue	% of recovered Y-90/g wet tissue Day 1	Day 3	Day 7
Liver	22.7%	36.7%	36.4%
Bone	24.0	42.1	25.1
Muscle	1.4	.4	.2
Spleen	6.0	10.3	33.1
Blood	.5	.6	.1
Kidney	6.0	10.6	1.5
Lung	2.0	2.2	2.4
Adrenal	5.9	5.8	1.2
Heart	3.3	1.6	.3
Brain	.1	.2	.5

able to estimate the relative contribution of activity in the individual tissues. Such high specific activities associated with bone, however, are suggestive that these colloids may be undergoing some dissociation within the animal body with subsequent deposition of "ionic" yttrium in bone substance in a manner such as that previously described(6,7).

Toxicity of radioyttrium hydroxide. Though a few animals survived a much larger dose, the mouse LD_{50} for this colloid appears to be 12-14 mc/kg of body weight. The small number of test animals does not permit a more precise evaluation of toxicity (Table II).

Excretion of radioyttrium hydroxide. Radioactivity is excreted very slowly, that is 0.5% in the first 24 hours, decreasing to 0.03% at 5 days (Table III). Thus, less than 1% of the administered dose is excreted during an interval of 5 days.

Distribution of radioyttrium colloids in man. Consideration of Table IV relative to tissue deposition of maltose-suspended yttrium hydroxide in man, and Table V concerning the deposition of carboxymethylcellulose-suspended yttrium phosphate in man, warrants certain observations. As with previously described radiocolloids, these products deposit primarily in the reticulo-endothelial tissues of the human body. Thus, marrow, spleen and liver accumulate appreciable quantities. To a lesser extent bone, kidney, and lungs also demonstrate radiocolloid uptake. Other tissues, including adrenal, heart, lymph nodes, and muscle, show little uptake. In these experiments, where bone and bone marrow could be successfully separated and assayed independently, it was noted that bone deposition was not a prominent feature in distribution of yttrium phosphate. It did, however, account for a greater portion of the distribution of yttrium hydroxide by comparison. Such results would tend to confirm the metabolic stability of yttrium phosphate and to a less extent the yttrium hydroxide, a conclusion which could not be reached on the basis of mouse data alone. With this rather limited group of subjects, no correlations were evident between size of dose administered, diagnosis, or time of survival after administration of radiocolloid and the particular tissue deposition observed in that individual patient.

TABLE VII. Tissue Deposition of YPO_4 – Y-90 in Mice after I.V. Administration.

Tissue	% of recovered Y-90/g wet tissue Day 1	Day 3	Day 7
Bone	57.9%	58.2%	50.6%
Liver	18.4	22.9	31.2
Lung	1.8	1.6	.8
Muscle	2.1	.7	.1
Blood	1.9	.1	.1
Kidney	9.1	3.4	2.0
Heart	1.1	.6	.3
Brain	.3	.0	.1
Spleen	2.2	8.9	14.5
Adrenal	—	2.2	2.4

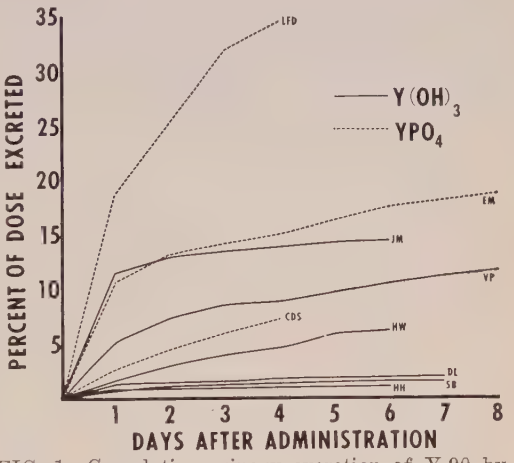

FIG. 1. Cumulative urinary excretion of Y-90 by man after i.v. colloidal $Y(OH)_3$ and YPO_4.

Urinary excretion of radioactivity by man. It is evident from Table III and Fig. 1 that urinary excretion of radioactivity by man exceeds that observed in mice quite ap-

preciably. Thus, of some 9 patients studied, only 3 showed total excretion of 2% or less during the first 5 days, while the remaining 6 patients demonstrated a cumulative excretion of 5 to 35% of administered dose during this same time interval. Quite probably this latter figure (35% in 4 days) was associated with gross hematuria in this particular patient. There seems no obvious correlation between tissue deposition in the individual patients and the excretory patterns shown by them.

Summary. 1. Simple technics have been described for preparation of 2 new colloids of radioyttrium $Y(OH)_3$ and YPO_4. Maltose has been employed as suspending agent for the former, carboxymethylcellulose for the latter. Both products have been demonstrated to have a small, uniform particle size, good emulsion stability, and low percentage dialyzable radioactivity. 2. The LD_{50} of $Y(OH)_3$ – Y-90 in mice was approximately 12 to 14 mc/kg. 3. Tissue deposition in mice and terminal human patients has been predominantly reticulo-endothelial in both. Evidence is suggestive that some metabolic dissociation occurs with subsequent deposition of yttrium in bone. 4. Urinary excretion of activity has been evaluated for mice and man.

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Normal Growth of Rat Mammary Glands During Pregnancy and Early Lactation.* (26365)

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Kirkham and Turner(1) first suggested desoxyribonucleic acid (DNA) may be employed as a quantitative index of mammary gland development under normal and experimental conditions. By this method, mammary gland proliferation has been observed to occur through pregnancy in the mouse(2, 3), and during lactation in the rat and mouse (3,4). This paper presents a more detailed study of mammary gland growth during pregnancy and early lactation in the rat.

Methods. Data were obtained from primiparous Sprague-Dawley-Rolfsmeyer rats on days 5, 10, 15, 18-20 of pregnancy and on days 1, 2, 3, and 4 of lactation. Days of pregnancy were determined by presence of sperm within the vagina. Six abdominal-inguinal mammary glands were removed from each rat and placed in a deep freeze for 4 days, extracted in 95% ethanol for 3-5 hours and in ether for an additional 3-5 hours. Dry, fat-free tissue (DFFT) was weighed and ground to a fine powder in a Wiley mill. DNA of a 25 mg aliquot of tissue was determined by method of Webb and Levy(5). Product of quantity DNA/mg DFFT and DFFT/100 g body weight (B.W.) was estimated as "total DNA".

Results. Mammary gland DNA/100 g B.W. of normal non-pregnant rats has been

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† Public Health Service Research Fellow of Nat. Cancer Inst.

TABLE I. Normal Growth of Rat Mammary Gland during Pregnancy and Early Lactation.

Stage of pregnancy or lactation	No. of animals	Body wt (g) mean	DFFT* (mg) mean	DNA (μg/mg DFFT)	Total DNA (mg/100 g B.W.)
Virgin control†	10	281	303	28.8 ± 1.0 §	3.11 ± .14
5th day of pregnancy	21	252.5‡	288	25.2 ± .88	2.83 ± .09
10th *idem*	21	255.0‡	363	33.0 ± 1.81	4.52 ± .35[2]
15th "	19	264.4‡	422	43.0 ± 1.89	6.76 ± .31[2]
18-20 " †	19	324.3‡	749	33.2 ± .80	7.63 ± .39[2]
1st day of lactation	11	245.3	584	35.6 ± .95	8.46 ± .77
2nd *idem*	10	247.5	659	37.7 ± 1.55	10.05 ± .52[3]
3rd "	8	268.6	789	37.1 ± 1.72	10.78 ± .34[4]
4th "	8	262.8	735	37.5 ± 1.26	10.45 ± .64[4]
5th " †	10	301.5	954	34.1 ± .73	10.80 ± .57[4]
14th " †	25	298.1	1240	26.3 ± .5	10.92 ± .43[4]

* Dry, fat-free tissue. † Data from Moon *et al.*(7) and Griffith & Turner(4). ‡ Corrected for wt of fetuses. § Mean and stand. error of mean.

[2] Signif. to day 5 of pregnancy at .1% level.
[3] Signif. to day 18-20 of pregnancy at .5% level.
[4] Signif. to day 18-20 of pregnancy at .1% level.

reported as 3.11 mg(6) and corresponding ovariectomized values as 3.05 mg(7). DNA was found to increase progressively from 2.83 mg at day 5 of pregnancy to 4.52 mg 6.76 mg, and 7.63 mg at days 10, 15, and 18-20 of pregnancy (Table I). DNA was significant ($P > .001$) between days 5 and 10; values at days 15 and 20 of pregnancy were significantly increased ($P > .001$) in comparison to the 5th day. Significance was also observed between days 10 and 15, but the increase from days 15 to 20 was not significant ($P < .05$).

DNA increased after parturition until day 2 of lactation and statistically remained at this level throughout lactation. DNA of day 2 of lactation was not significant ($P < .05$) but days 3, 4, and 5 are highly significant ($P > .001$) to the latter days of pregnancy. There is no significant difference between the values throughout lactation except in comparison to day 1.

The decrease of DFFT during early lactation can not be explained at present time.

Discussion. Data presented here indicate lobule-alveolar development of mammary gland in rat continues throughout pregnancy and early lactation. Considering extent of mammary growth at end of pregnancy, only 81.9% of maximum development occurred by the 15th day of pregnancy; the remaining 18.1% occurred during the last 5 days of gestation. Similar results were observed with pregnant mice(2,3).

Data presented previously(4) indicated the mammary glands do not attain maximum cellular development until days 5 or 10 of lactation. Extending study to include days 1, 2, 3, and 4 of lactation, DNA was found to increase significantly by day 3 of lactation.

When extent of mammary growth at day 14 of lactation was considered as 100%, the mammary gland only attained 59.3% of its maximum development by end of pregnancy. The remaining 40.7% occurred during early lactation. The present findings which are in conflict with older views of mammary development are substantiated by recent investigations(3,8). Estimation of total DNA of mouse mammary glands revealed 76.3% of total development was obtained during pregnancy; 23.7% occurred after parturition(3). Greenbaum and Slater(8) determined the nucleoprotein content of homogenates of rat mammary tissue. Between days 1 and 3 of lactation DNA-P level was doubled in 2 ml of a 1:10 tissue suspension. Authors suggested a wave of mitosis occurs in gland at parturition. By the use of colchine, a wave of cell division was observed approximately 30 hr after parturition, and number of nuclei/mg wet tissue of mouse mammary gland was found to increase approximately 120,000 from day 1 to 2 of lactation.

Summary. Previous morphological studies were interpreted as indicating complete lobule-alveolar development by first ½-⅔ of

pregnancy. By estimation of DNA, a study of rat mammary gland growth was conducted through pregnancy and early lactation. Present data show 20.9% of total growth occurs during the first 10 days of pregnancy, 59.3% by the 20th day of pregnancy, and development is 98.3% complete by the 3rd day of lactation.

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Absorption of Potassium Iodide from Gastro-Intestinal Tract.* (26366)

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Prompt excretion of orally administered iodide by the salivary gland provides a simple measure of the rapidity of its absorption from the gastro-intestinal tract. Salivary iodide excretion is clearly altered in a variety of clinical states in man(1), but the site of its absorption is controversial(1,2,3,4,5,6). In view of its possible utility as a measure of gastrointestinal absorption, it seemed desirable to determine what organ or organ system is involved in its absorption.

Methods. 1. *Human*: The potassium iodide absorption test(1) was conducted as follows: Subjects were given a solution of 0.25 g of potassium iodide in water, *per os*, following a 14- to 16-hour fast. Their mouths were then rinsed. Saliva was collected every 2 minutes and tested for presence of iodine (1 ml of saliva added to 1 ml of a 1% starch solution containing 4 drops of a 10% $FeCl_2$ solution; this test is positive for as little as .0027 mg of iodine). Ten normal subjects were found to have iodine present in their saliva in from 6 to 14 minutes, well within the normal limits determined by Heath(1). Seven of these subjects were retested similarly, except that $BaSO_4$ was added to the potassium iodide solution in sufficient quantity to produce a moderately thick suspension, and the studies were done under fluoroscopic control. The subjects were placed on a fluoroscopy table and were rotated so that all portions of the stomach were exposed to the iodide-containing radio-opaque medium. They then were placed in a supine position, rotated slightly to the left. Saliva was collected and tested for iodine every 2 minutes, and fluoroscopic observations were made at these same intervals. One of the subjects in this group was restudied (after an interval of 3 days) with Urokon® (50%) as the opaque substance to obviate the possibility of physical interference with absorption by $BaSO_4$. The saliva of all of the above subjects contained no iodide until some of the radio-opaque medium had been seen to pass into the duodenum (Table I).

A solution of potassium iodide was instilled, *via* a tube, directly into the duodenum of 2 of the previously tested subjects. Iodide was detected in their saliva in 2 to 4 min-

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TABLE I. Potassium Iodide Absorption in Humans.

Potassium iodide	Min. between intake of KI solution (including contrast media) and (a) Duodenal appearance of barium	(b) Salivary appearance of iodine	Time from duodenal to salivary appearance
	Min.		
In barium sulfate	22	24	2
	12	15	3
	22†	25	3
	*	—	—
	*	—	—
	37†	40	3
	35†	39	4
In diodrast†	*	—	—
In barium sulfate instilled directly into duodenum	Direct instillation	3	3
	"	4	4
In barium sulfate given after 7.5 mg I.V. probanthine	110	114	4
	120	123	3
	130	133	3

* Test discontinued after 20 min.
† Patients studied twice.

utes. This corresponds to the results obtained when potassium iodide (.25 g in 20 ml sterile distilled H_2O) was given intravenously to 4 alcoholic patients(7). Three subjects were given .25 g of potassium iodide in a $BaSO_4$ mixture, *per os,* after they received intravenous probanthine which was given to delay gastric evacuation. Saliva of these subjects was negative until there was fluoroscopic evidence of $BaSO_4$ in the duodenum.

2. *Wistar strain male rats* weighing between 147 to 181 g were used. Animals were secured to a board; their abdomens were shaved and infiltrated with 1% xylocaine hydrochloride. Group I (11 rats): A midline incision was made and a polyvinyl tube was passed perorally into the stomach. A loose ligature was placed at the cardia, to be tied at termination of experiment, and an occluding ligature was placed at the pylorus. (All major vessels were avoided.) The stomach then was evacuated *via* the tube and washed with 5 ml of distilled water. Two ml of a 25% solution of potassium iodide (in .01N HCl) was injected into the stomach. The tube then was removed and abdominal incision closed. The animals were sacrificed after a 1-hour absorption period. The stomachs were removed and the contents quantitatively extracted with demineralized water.

Group II (12 rats): A midline incision was made and a polyvinyl tube was passed perorally and manipulated into the duodenum. A loose ligature was placed at the pylorus (excluding all major vessels) and 2 ml of a 25% solution of potassium iodide (in .01N HCl) was slowly (1-1½ min) injected into the intestine. The tube then was removed, the pyloric ligature tied to prevent flow from the stomach, and the abdominal incision was closed. Animals were sacrificed following one hour of absorption. The entire intestinal tract, excluding the stomach, was removed and the contents quantitatively extracted and analyzed for retained iodine by protein bound iodine methods(8). An average of 55 mg potassium iodide/100 g rat/hr was absorbed from the intestine (Table II). The difference is highly significant ($P > .01$).

Discussion. The present studies support the contention of several authors that the principal site of absorption of iodide is the small intestine. Heath and Fullerton(1) studied absorption of iodide in human subjects, using the method employed in this present study, and found that iodine could be detected in saliva of normal subjects in 6 to 15 minutes after oral ingestion, while its appearance was markedly delayed in patients with scurvy, myxedema, pernicious anemia and alcoholic cirrhosis. They felt that this delay reflected *intestinal* malabsorption on the basis of the work of Cohn(2), Eisenmann (3) and others. Cohn(2) assessed absorption of iodine in isolated segments of the intestine of anesthetized dogs. He found that 63% of potassium iodide placed in the colon

TABLE II. Potassium Iodide Absorption in Rats.

No. rats	Avg wt, g	Potassium iodide inj., mg	Potassium iodide absorbed mg	%	mg/100 g /rat/hr
11	164	570	85	14.9	55 ± 21
12	163	650	320	48.9	197 ± 36

and from 74 to 80% of the potassium iodide placed in the jejunum was absorbed, while no more than 31% of the iodide placed in the stomach was absorbed. The difficulty in recovering the contents of the stomach, this author suggested, might have caused a loss which would place the percent absorbed higher than the actual amount absorbed. In support of the absorptive role of the stomach are the findings of Rankin(4), Henning(5) and Bertrand(6). Rankin(4) instilled potassium iodide into the rumen of sheep and detected iodine in the saliva in 4 minutes, concluding that the stomach was a principal site of iodine absorption. Henning(5) and Bertrand(6) found that patients with chronic gastritis and gastric ulcer excreted salivary iodine more rapidly than normal subjects, assuming that potassium iodide was absorbed in the stomach.

The difficulty in determining the exact time of pyloric passage may account for the discrepancy between our results and those of Henning(5) and Bertrand(6). Henning assumed that by placing the subject in the left lateral decubitus position, negligible amounts of gastric contents would enter the duodenum, while Bertrand determined time of passage using an air-fluid contrast technic under fluoroscopic control. In the present study, the use of radio-opaque media permitted more accurate determination of the passage of small quantities of potassium iodide into the small intestine. Also, the time elapsed between passage into the duodenum and salivary appearance of iodide agreed closely with the time required for detection of salivary iodide when potassium iodide was instilled directly into the duodenum.

Summary. Potassium iodide mixed with radio-opaque media was administered orally to human subjects studied under fluoroscopic control. Iodide appeared in saliva of these subjects 2-4 minutes after mixture passed into the duodenum. Iodide instilled directly into the duodenum of 2 subjects was detected in their saliva 3-4 minutes later. 14.9% of the iodide placed in the stomach and 48.9% of the iodide placed in the duodenum of rats was absorbed within one hour. It appears that the small intestine is the principal site of absorption in humans and in rats.

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The Local Tissue Reaction in Rabbits to Gelfoam Implants Containing Desmosterol or Cholesterol.*† (26367)

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A recently developed compound, Triparanol (MER-29), has been shown to inhibit biosynthesis of cholesterol(1). It has been further demonstrated that the biosynthesis of cholesterol is probably inhibited at the step which requires reduction of the side chain double bond of 24 dihydrocholesterol, with a resultant increase in serum levels of desmosterol (precursor to cholesterol)(2). MER-29 is at present being studied in hu-

* The desmosterol used in this study was supplied by Wm. S. Merrell Co., Cincinnati, Ohio.

† MER-29 is trade name of compound produced by Wm. S. Merrell Co.

mans as to its value as a serum cholesterol lowering agent. Since the serum level of desmosterol appears to be increased under these circumstances, it is a matter of importance to know the effects of desmosterol on mammalian tissues. It is known that extracellular deposits of cholesterol or the esters of cholesterol excite an inflammatory reaction with fibrosis and foreign body granuloma in the body tissues. The basic ingredients of this reaction are essentially similar, whether the cholesterol is found in the subintima of blood vessels or elsewhere. The purpose of this paper is to report on the reaction to desmosterol that was introduced into the extracellular tissues experimentally in the rabbit. The technic employed in this study has been described(3).

Materials and methods. Standardized pieces of sterile absorbable gelatin (gelfoam) sponges each weighing approximately 3 mg were placed in 1 cc of ether containing 4.3 mg of desmosterol (the entire amount of desmosterol available for this study). The ether was then allowed to evaporate. The 6 gelfoam sponges were estimated to contain approximately 0.7 mg of desmosterol each. These desmosterol-containing gelatin sponges were implanted in the subcutaneous region through a small surgical incision in the abdominal surface of 6 rabbits. Also implanted simultaneously in each one of the rabbits, at separate sites, were similar size gelfoam sponges saturated with cholesterol and gelfoam sponges that were not impregnated with any material. Each rabbit, therefore, had 3 subcutaneous implants with various gelfoam sponges. The rabbits were males of equivalent weight and age and maintained on stock diets. After 14 days the implants and surrounding tissue were excised from these rabbits, fixed in formalin and sections were prepared that were stained with hematoxylin and eosin. The number of implants was limited because of the small amount of desmosterol available.

Findings. The reaction to the gelfoam-desmosterol implants revealed an intense acute inflammatory response with considerable cellular necrosis. This reaction permeated the entire extent of the sponges of all 6 rabbits. Minimal to moderate granulation tissue surrounded each one of these implants. The histologic reaction to the gelfoam cholesterol implants revealed a similarly intense inflammatory reaction. However, in this situation the macrophages predominated. Granulation tissue in amounts similar to those seen in the gelfoam desmosterol implants was present. The histologic reaction to the control gelfoam sponge implants revealed no inflammatory reaction and only slight fibroblastic proliferation surrounding the sponges.

Summary and conclusions. It would appear from this limited study in rabbits that desmosterol lying free in extra-cellular sites is not innocuous, in that it excites an intense inflammatory reaction in the subcutaneous site of implantation. The fate of desmosterol and its distribution in the body tissues is not yet known. Whether or not desmosterol may appear freely in the extra-cellular tissues of blood vessel walls as is the case with cholesterol is also not known. Because of the fact that desmosterol does incite an intense inflammatory reaction, its fate and distribution in the body should be determined before MER-29 can be considered to be a relatively safe serum cholesterol lowering agent.

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